Functional and Structural Uncoupling of the Angiogenic and Enzymatic Inhibitory Activity of TIMPs

Loop 6 of TIMP-2 is a Novel Inhibitor of Angiogenesis

by

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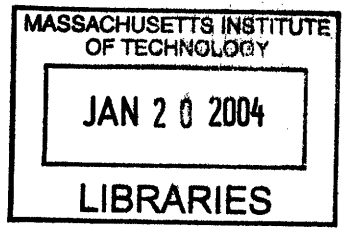

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Abstract

Tissue inhibitors of metalloproteinases (TIMPs) regulate tumor growth, progression and angiogenesis in a variety of experimental cancer models and in human malignancies. However, numerous studies have revealed important differences between TIMP family members in their ability to inhibit angiogenic processes *in vitro* and angiogenesis *in vivo* despite their universal ability to inhibit matrix metalloproteinase (MMP) activity. To address these differences, structure-function studies were conducted to identify and characterize the anti-angiogenic domains of TIMP-2, the endogenous MMP inhibitor that uniquely inhibits capillary endothelial cell (EC) proliferation and angiogenesis *in vivo*. Only the carboxy-terminal domain of TIMP-2 (T2C) and not the MMP-inhibitory N-terminal domain (T2N), inhibited capillary EC proliferation. Although both T2N and T2C inhibited embryonic angiogenesis, only T2C potently inhibited mitogen-stimulated angiogenesis. These findings demonstrate that TIMP-2 possesses two distinct types of anti-angiogenic activities which can be uncoupled from each other. The anti-proliferative activity of T2C was further mapped to the 24-amino acid peptide, Loop 6, which proved to be a potent inhibitor of both embryonic and mitogen-stimulated angiogenesis *in vivo*.

Initial studies into the mechanism(s) by which Loop 6 inhibits angiogenesis revealed that the anti-proliferative effects of Loop 6 are due, at least in part, to the inhibition of cell cycle progression and not to the induction of apoptosis. This inhibition was associated with increased levels of cell cycle inhibitor p27. Although Loop 6 did not compete with bFGF for binding to its receptor, five potential cell surface complexes were observed in crosslinking studies of capillary EC treated with ^{125}I-labeled T2C or Loop 6.

Finally, given the high degree of homology between TIMP-2 and TIMP-4, we hypothesized that TIMP-4 might share anti-proliferative and MMP inhibition-independent anti-angiogenic activities with TIMP-2. Our results demonstrate that although TIMP-4 inhibits capillary EC migration, it does not inhibit capillary EC proliferation. Furthermore, TIMP-4 did not result in significant inhibition of embryonic angiogenesis in the CAM. These results suggest that TIMP-2 is unique among TIMP family members in its ability to inhibit angiogenesis via two distinct pathways. One of these activities, housed within Loop 6, results in the potent inhibition of angiogenesis *in vivo*.

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Preface

The research presented in this dissertation is aimed at understanding the anti-angiogenic effects of TIMPs and how they relate, or not, to the direct inhibition of MMP activity. To date, TIMP-2 has been shown to be unique among the TIMP family members, in that it is the only TIMP capable of inhibiting capillary endothelial cell proliferation, a key process in the formation of new capillaries known as angiogenesis. Another angiogenic process, capillary endothelial cell migration, is inhibited by all TIMPs tested to date (TIMPs –1, -2, and –3), presumably based on their ability to directly inhibit MMP activity. Based on these data, it has largely been assumed that all TIMPs are capable of inhibiting angiogenesis, despite the fact that not all TIMPs have similar effects on other angiogenic processes, namely capillary EC proliferation and despite the fact that TIMP-4 has never been specifically tested for its ability to inhibit angiogenic processes *in vitro* or angiogenesis *in vivo*.

Given that all TIMPs have been shown to inhibit MMP activity equally well, the ability of TIMP-2 to inhibit capillary EC proliferation has been proposed to constitute a second function that is independent of MMP inhibitory activity. One of the goals of this thesis research was to identify the TIMP-2 domain(s) responsible for the ability of TIMP-2 to inhibit angiogenesis, both via the direct inhibition of MMP activity and via the inhibition of capillary EC proliferation. These studies constitute the main focus of the work presented in this dissertation, are described in detail in Chapters 2 through Chapter 5, and have recently been reported (Fernandez et al., 2003).

Having isolated the anti-angiogenic domain of TIMP-2 that inhibits *in vivo* angiogenesis independent of MMP inhibition, the next major goal of this research was to begin to examine the possible mechanism of action of this domain. These studies, described in Chapter 6 of this dissertation, focus on understanding the set of events that ultimately result in the inhibition of EC proliferation *in vitro* and angiogenesis *in vivo*.

Since it has largely been assumed that all MMP inhibitors inhibit angiogenesis, it has also been assumed that TIMP-4 is capable of inhibiting angiogenesis. However,

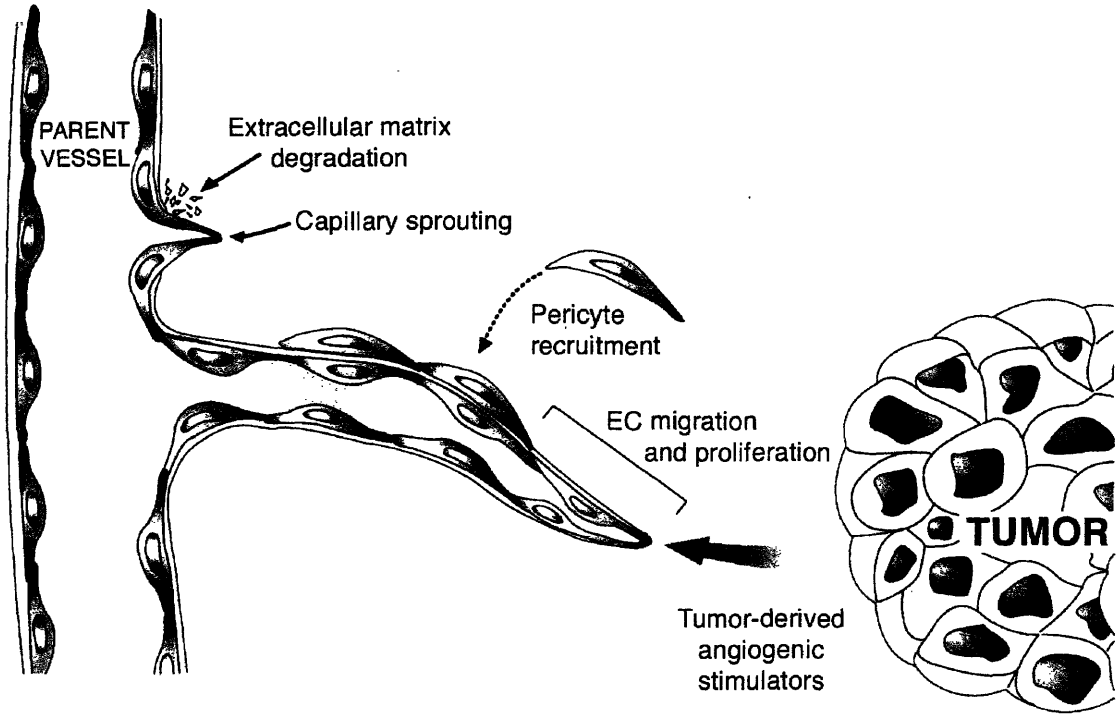
TIMP-4 had yet to be tested for its anti-angiogenic effects either *in vivo* or *in vitro*. Because the main goal of this thesis was to understand the anti-angiogenic effects of TIMPs, a third focus of this research included assessing the ability of TIMP-4 to inhibit angiogenic processes *in vitro* and angiogenesis *in vivo*. These results are presented in detail in Chapter 7 of this dissertation.

Chapter 1: Background

Section A: Angiogenesis

Angiogenesis, the process of new capillary vessel formation from preexisting venules, is a key event in tumor growth. Neovascularization of the tumor supports its growth and may provide a route of escape for metastatic cells. During angiogenesis, normal capillary endothelial cells (EC) are stimulated by an angiogenic mitogen such as fibroblast growth factor (FGF) or vascular endothelial growth factor (VEGF) to escape the parent venule, to proliferate and then to migrate towards the source of stimulus (Figure 1). The ability of capillary EC to remodel the extracellular spaces during these processes is largely dependent on the concerted activity of matrix degrading enzymes and their inhibitors. Complete formation of a new mature vessel is achieved after lumen formation and the recruitment of pericytes to promote endothelial cell differentiation and the formation of a mature vessel (Folkman, 1971; Ausprunk and Folkman, 1977; Klagsbrun and Moses, 1999; Folkman, 2002).

Angiogenesis occurs rarely under normal physiological conditions, such as wound healing and in reproduction and development, and is dependent on the balanced activity of positive and negative regulators of angiogenesis. Positive regulators include the angiogenic mitogens bFGF and VEGF, which stimulate the proliferation and migration of capillary EC (Shing et al., 1984; Brown et al., 1989; Leung et al., 1989), as well as proteolytic enzymes, such as the matrix metalloproteinase (MMPs), whose activity is required in order for capillary EC to degrade the extracellular matrix around them allowing them to migrate towards a source of stimulus (Klagsbrun and Moses, 1999;



From Wu, I. and Moses, M.A., *Current Oncology Reports*, 2(6):566-71, 2000

Figure 1. Angiogenesis. The process of angiogenesis is initiated by an angiogenic mitogen which stimulates endothelial cells in a nearby vessel to degrade the basement membrane surrounding the parent vessel, and to migrate and proliferate towards the source of stimulus. Lumen formation and subsequent perycite recruitment complete the new vessel maturation.

Stetler-Stevenson, 1999; Folkman, 2002). MMPs, the rate-limiting enzymes in new capillary formation, are discussed in more detail below.

Since it was first postulated that the inhibition of angiogenesis might represent a valuable clinical tool in the treatment of various pathological conditions which are dependent on angiogenesis (Folkman, 1971; Folkman, 2001) such as cancer, macular degeneration and arthritis, much attention has been placed on identifying inhibitors of angiogenesis. Endogenous proteins known to negatively regulate angiogenesis include, Thrombospondin (Vogel et al., 1993; Iruela-Arispe et al., 1999), Troponin I (Moses et al., 1999), and the tissue inhibitors of metalloproteinases (TIMPs) (Moses et al., 1990; Moses and Langer, 1991; Murphy et al., 1993; Johnson et al., 1994; Anand-Apte et al., 1997), which are discussed further below. Interestingly, a number of inhibitors recently identified are, in fact, fragments of larger proteins that may or may not contain the same activity. Examples of this include angiostatin and endostatin, which are fragments of plasminogen and collagen XVIII, respectively (O'Reilly et al., 1994; O'Reilly et al., 1997). Counterintuitively, MMPs have also been shown to release both angiostatin and endostatin from the parent molecule (O'Reilly et al., 1999; Wen et al., 1999), suggesting that MMPs may act as negative regulators of angiogenesis in some instances. As further discussedmbelow, similar contradictory effects have been described for their inhibitors, the TIMPs (Guedez et al., 2001; Yan and Moses, 2001; Jiang et al., 2001).

Since deregulated proteolytic activity is a hallmark of processes such as angiogenesis, tumor growth and metastasis (Liotta et al., 1991a; Liotta et al., 1991b), the role that MMPs and their inhibitors play in angiogenesis has been the focus of intense research.

Section B: Matrix metalloproteinases

An accumulating body of evidence suggests that the remodeling of the extracellular matrix during angiogenesis is accomplished largely through the activity of the matrix metalloproteinases (MMPs) (Matrisian et al., 1994; Stetler-Stevenson et al., 1996; Birkedal-Hansen, 1995; Moses, 1997). MMPs form a large family of metal-dependent proteolytic enzymes, which have been classified based on the extracellular matrix components that they degrade (Table I). There are four major classes of MMPs: collagenases, gelatinases, stromelysins, and membrane-bound MMPs. Normal matrix turnover is dependent on the regulation of MMPs at various levels: at the translational level, in the activation from its inactive proenzyme form, and by the activity of their specific inhibitors the tissue inhibitors of matrix metalloproteinase (TIMPs) (Matrisian et al., 1994; Stetler-Stevenson et al., 1996; Birkedal-Hansen, 1995; Moses, 1997; Nagase and Woessner, 1999) (Figure 2).

MMPs have been clearly implicated in the promotion angiogenesis as well as tumor growth and metastasis. It is the proteolytic activity of these enzymes at the leading edge of migrating cells that allows for the cell's advance through the extracellular spaces during angiogenesis. For example, MMP-2 activity has been shown to be concentrated at the invadopodia of various cell lines (Monsky et al., 1993; Brooks et al., 1996). The collective activities of MMPs have been shown to degrade all known components of the ECM, which provides tissue structure as well as a scaffold for cell architecture (physical barrier). MMP activity is therefore required for cell migration and invasion. This activity is also required for a cancer cell to be able to invade a nearby blood vessel and

Table 1. Matrix metalloproteinases

Subfamilies	Trivial name	MMP number	Molecular mass (kDa)		Prototypical ECM Substrates
			L	A	
Collagenases	Collagenase-1	MMP-1	52	42	Collagen I, II, III, VII, VIII, X, XI; gelatin I, entactin/nidogen, fibronectin, laminin, perlecan vitronectin, aggrecan
	Collagenase-2	MMP-8	85	64	Collagen I, II, III; gelatin, entactin, aggrecan, tenascin
	Collagenase-3	MMP-13	52	42	Collagen I, II, III, VI, IX, X, XIV; gelatin I, fibronectin, vitronectin, aggrecan, osteonectin
	Collagenase-4	MMP-18	53	42	Collagen type I, II, III; gelatin
Gelatinases	Gelatinase A	MMP-2	72	66	Collagen I, III, IV, V, VII, X, XI; gelatin I, fibrillin, fibronectin, elastin, vitronectin, aggrecan, osteonectin, tenascin, laminin-5
	Gelatinase B	MMP-9	92	84	Collagen IV, V, XI, XIV; gelatin I, decorin, elastin, fibrillin, laminin, vitronectin, aggrecan
Stromelysins	Stromelysin-1	MMP-3	57	45	Collagen III, IV, V, VII, IX, X, XI; gelatin I, decorin, elastin, entactin/nidogen, fibrillin, fibronectin, laminin, vitronectin, aggrecan, osteonectin
	Stromelysin-2	MMP-10	54	44	Collagen III, IV, V; gelatin I, elastin, fibronectin, aggrecan
MT-MMPs	MT-MMP1	MMP-14	66	54	Collagen types I, II, III; gelatin, fibronectin, vitronectin, aggrecan
	MT-MMP2	MMP-15	72	60	Proteoglycan
	MT-MMP3	MMP-16	64	53	Collagen type III, fibronectin
	MT-MMP4	MMP-17	57	53	Gelatin, fibrin/fibrinogen
	MT-MMP5	MMP-24	73	62	Fibronectin, proteoglycans, gelatin
	MT-MMP6	MMP-25	63	62	Collagen type IV, gelatin, laminin-1, fibronectin, proteoglycans, fibrin/fibrinogen
Matrilysins	Matrilysin-1	MMP-7	28	19	Collagen I, IV, decorin, elastin, fibrillin, fibronectin, laminin, decorin vitronectin, aggrecan, osteonectin,
	Matrilysin-2	MMP-26	29	19	Collagen type IV, gelatin, fibronectin, fibrin/fibrinogen
Other MMPs	Stromelysin-3	MMP-11	64	46	Fibronectin, laminin, aggrecan
	Metalloelastase	MMP-12	54	22	Fibronectin, elastin, laminin, proteoglycan, fibrin/fibrinogen
	RASI-1	MMP-19	54	45	Collagen type IV, gelatin, laminin, fibronectin, fibrin/fibrinogen
	Enamelysin	MMP-20	54	22	Amelogenin, aggrecan
	XMMP	MMP-21	70	53	No substrates defined
	CMMP	MMP-22	51	43	No substrates defined
	CA-MMP	MMP-23	44	31	Gelatin
		MMP-27			No substrates defined
	Epilysin	MMP-28	59	45	No substrates defined

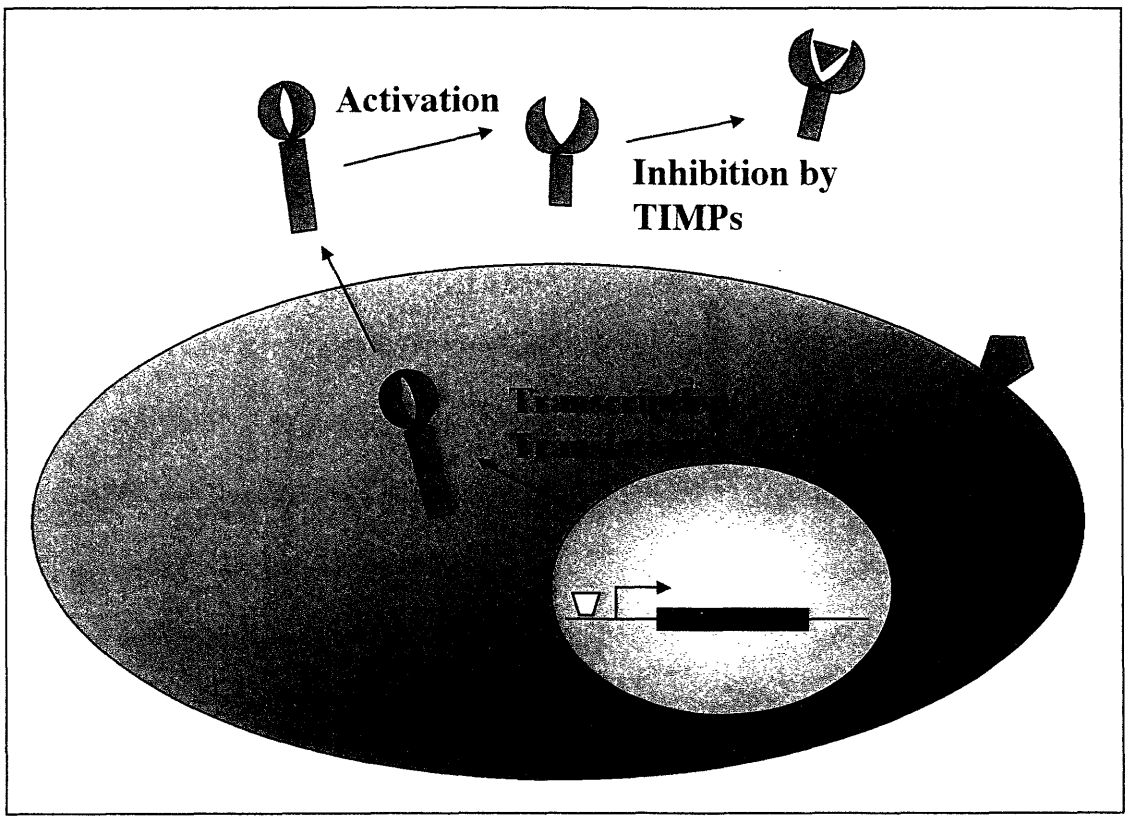


Figure 2. Regulation of MMP activity. The first level of MMP regulation is in the transcription and translation of the inactive pro-enzymes. Activation is achieved by the cleavage of the pro-domain. Once active, MMP activity can be inhibited by the direct binding of one of its endogenous inhibitors, the TIMPs, to the active site of the enzyme.

extravasate at a distant site and invade the distant tissue in order to seed a new metastasis. It has also been suggested that the proteolytic activity of MMPs might allow for the release of angiogenic mitogens deposited in the matrix, and, interestingly, MMPs have been shown to cleave membrane bound growth factors (Suzuki et al., 1997; Yu and Stamenkovic, 2000; Hashimoto et al., 2002).

Recent studies have demonstrated that MMPs are also involved in the angiogenic switch. In a model of tumor progression, which reliably recapitulates the earliest events associated with the onset of neovascularization, our lab has shown that MMP-2 is required for the angiogenic switch (Fang et al., 2000). In this study, both MMP-2 protein and activity were upregulated approximately three-fold in angiogenic tumors compared to pre-angiogenic tumors. Other have since shown that MMP-9 might be a regulator of the angiogenic switch in a pancreatic tumor model (Bergers et al., 2000), further confirming the pro-angiogenic role of MMPs. Our group and others have shown that MMPs are upregulated during tumor development in a variety of different models and that MMP activity is necessary for many of the stages of tumor progression including angiogenesis, migration and invasion of both endothelial cells and metastatic tumor cells. Moreover, angiogenic mitogens, such as bFGF and VEGF, have been shown to stimulate the production of MMPs by capillary EC (Mignatti et al., 1989; Unemori et al., 1992; Lamoreaux et al., 1998). These findings strongly suggest that MMP activity is critical, not only to the onset of angiogenesis, but to the maintenance of the growing vascular bed which in turn supports tumor growth and which might allow for the escape of tumor cells that give rise to metastasis. Taken together, these data support the conclusion that MMP activity is one of the earliest and most sustained events in angiogenesis and tumor

growth. As a result, proteolytic enzymes, based on their overproduction and their activity per se, might find their way into body fluids.

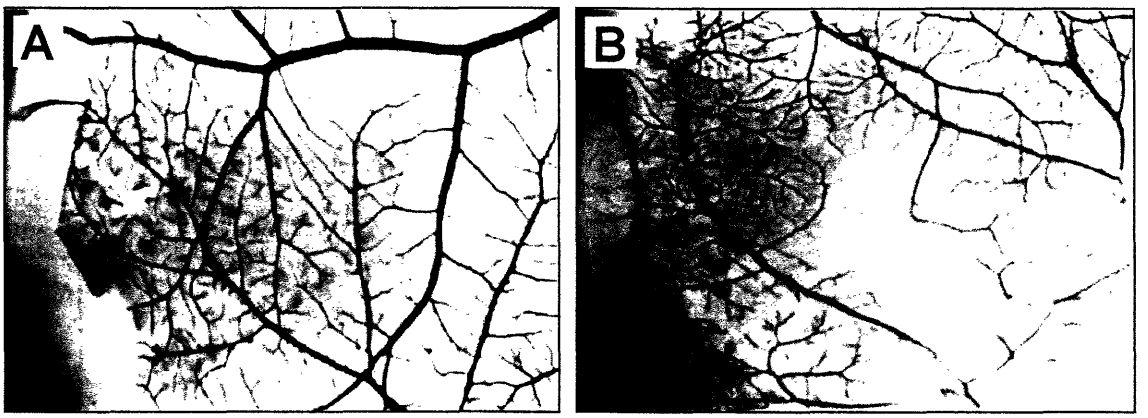
In fact, our lab has also shown that MMPs found in the urine of cancer patients are independent predictors of disease status (Moses et al., 1998). These studies demonstrate that three MMP species, MMP-2, MMP-9 and species of "high molecular weight" (Mr >140-150 kDa) MMPs were reproducibly detected and correlated with disease status and were not found in the urine of healthy age-matched, sex-matched controls or in the urine of patients which showed no evidence of disease at time of sampling.

Taken together, these data underline the importance of MMPs in cancer and as pro-angiogenic molecules. Based on these findings, MMP inhibition has long been thought to represent an important target for cancer therapy. In fact, it has been shown that a shift in the proteolytic balance in favor of the inhibitors would effectively block angiogenesis (Moses et al., 1990; Moses and Langer, 1991; Stetler-Stevenson et al., 1996; Moses, 1997; Stetler-Stevenson, 1999).

Section C: Tissue inhibitors of matrix metalloproteinases

Our lab was the first to show that an inhibitor of matrix metalloproteinases could also inhibit angiogenesis *in vivo* (Moses et al., 1990) (Figure 3). Since then, the therapeutic potential of TIMPs has been attributed only to their ability to directly inhibit MMP activity.

There are four TIMPs cloned to date, TIMP-1, TIMP-2, TIMP-3 and TIMP-4 (Docherty et al., 1985; Stetler-Stevenson et al., 1990; Pavloff et al., 1992; Greene et al.,



Moses, M.A., et al., *Science*, 248: 1408-1410, 1990

Figure 3. A cartilage-derived TIMP (CDI) inhibits angiogenesis *in vivo.* Methylcellulose discs containing either control (A) or CDI (B) were applied to the chorioallantoic membrane of 6 day old chicks. After 48 hours of incubation, the eggs were examined under a dissecting scope (60X).

1996). The primary structure of the TIMP family members is highly conserved, with all TIMP family members sharing approximately 45% overall homology (25% identity). TIMP-1 shares the highest homology with TIMP-3, while TIMP-2 shares a higher degree of homology with TIMP-4 (Table II). Sequence homology of all four TIMPs includes 12 conserved Cys residues that form disulfide bonds, in turn giving rise to a six loop secondary structure (Williamson et al., 1990; Nagase and Brew, 2002) (Figure 4). TIMPs inhibit MMPs by binding to the enzyme's active site, forming tight (K_i < 1 nM) non-covalent enzyme/inhibitor complexes in a 1:1 stochiometric ratio. This binding is thought to occur in two stages, where the initial binding is reversible, but gives way to a tight binding of the first cysteine residue of TIMPs to the Zn^{2+} in the enzyme's catalytic site to form the inhibited complex (Cawston et al., 1983; Welgus et al., 1985; Murphy et al., 1989).

Structurally, TIMPs have been classically divided into two domains based on deletion mutagenesis studies, the N-terminal domain, comprised of the first three disulfide-bonded loops, and the C-terminal domain, comprised of the last three disulfide-bonded loops (Murphy et al., 1991; Willenbrock et al., 1993; Nguyen et al., 1994). These studies and others showed that the N-terminal domain of TIMPs is sufficient to inhibit MMP activity, and therefore represent a minimized form of the inhibitors (Williamson et al., 1994a; Williamson et al., 1994b). These truncated proteins have been shown to bind to the catalytic sites of MMPs by both biochemical approaches as well as in crystal structures of enzyme/inhibitor complexes (Murphy et al., 1991; O'Shea et al., 1992; Tolley et al., 1993; Butler et al., 1999; Fernandez-Catalan et al., 1998; Muskett et al.,

1998). Importantly, the structure of the N-terminal domain of either TIMP-1 or TIMP-2 is not affected by the C-terminal domain (Nguyen et al., 1994), suggesting that these two domains can function independent of each other.

Although all four TIMPs have been shown to inhibit MMPs equally well, some minor differences in relative affinity for various MMPs exist from one TIMP to another. For example, TIMP-1 preferentially binds to MMP-9 and stromelysin, whereas TIMP-2 preferentially binds to MMP-2 (Nguyen et al., 1994; Olson et al., 1997). Interactions between the C-terminal domain of TIMP-2 and the PEX domain of MMP-2 are thought to stabilize the enzyme inhibitor complex (Howard and Banda, 1991; Willenbrock et al., 1993), as well as promote pro-MMP-2 activation in a trimeric complex with MT1-MMP (Strongin et al., 1995; Hernandez-Barrantes et al., 2000). Involvement of TIMP-2 in the cell surface activation of pro-MMP-2 is discussed further below.

Despite their universal ability to inhibit MMP activity, TIMPs have been shown to be multi-functional proteins (Brew et al., 2000; Baker et al., 2002). For example, TIMP-3 has been shown to induce apoptosis of various cell lines (Smith et al., 1997; Baker et al., 1999; Bond et al., 2000), while TIMP-1 has been shown to inhibit apoptosis (Guedez et al., 1998a; Guedez et al., 1998b; Li et al., 1999). In apparently contradictory reports, TIMP-4 has been shown to induce apoptosis of transformed cardiac fibroblasts (Tummalapalli et al., 2001) but to result in a decrease in breast cancer cell apoptosis *in vitro* and *in vivo* (Jiang et al., 2001). Similarly, TIMP-2 has been reported to either stimulate or inhibit programmed cell death (Lim et al., 1999; Brand et al., 2000; Bond et al., 2000). Additionally, both TIMP-1 and TIMP-2 have been shown to possess erythroid potentiating activity (Gasson et al., 1985; Stetler-Stevenson et al., 1992).

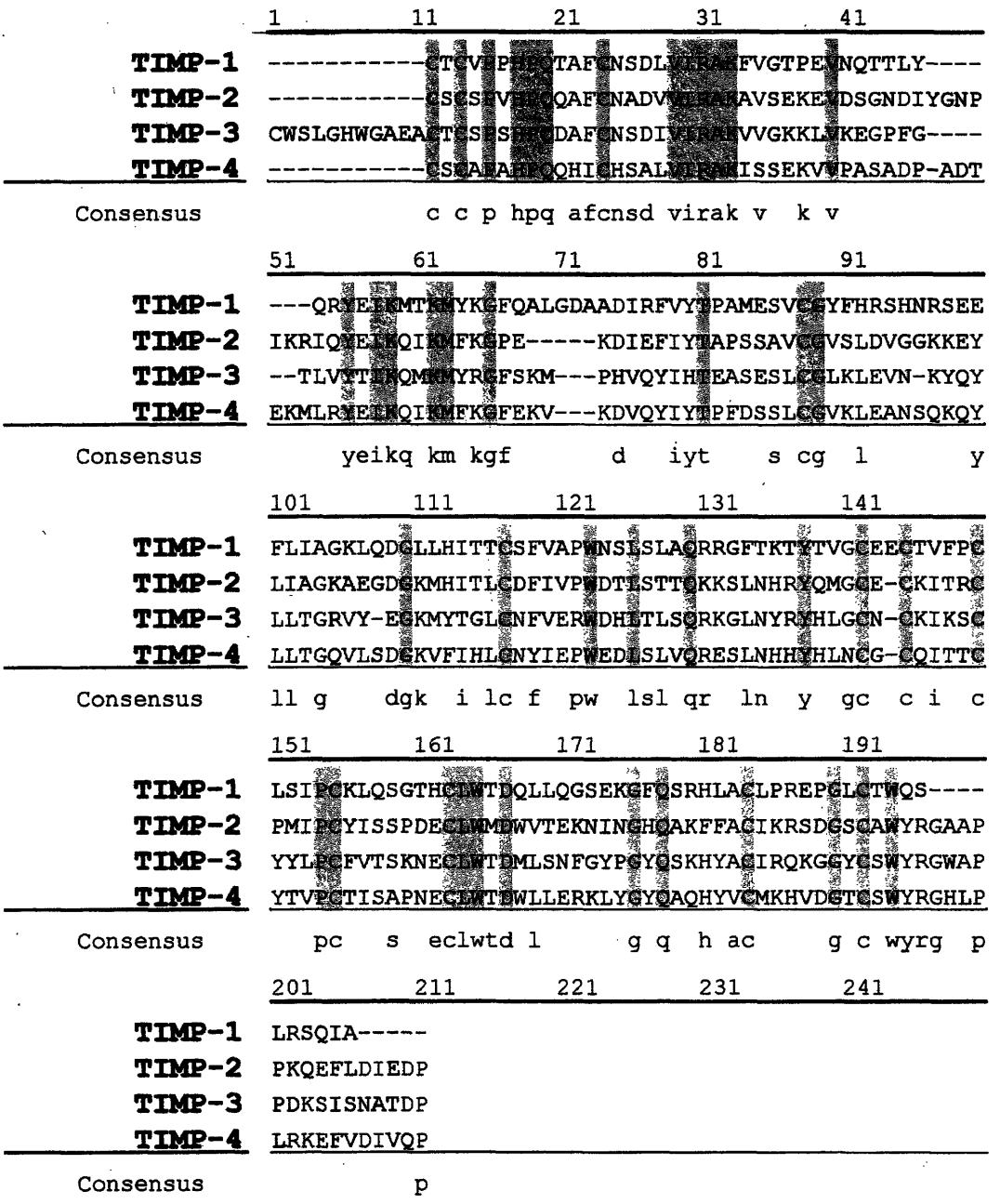


Table II. Sequence alignment of TIMPs. The amino acid sequences comprising human TIMPs 1-4 are shown. Residue 1 in each case is the first cysteine of the mature protein. Conserved residues are highlighted in yellow and residues conserved between two or more TIMPs are shown in blue. Residues conserved in three or more TIMPs are indicated below the sequence alignment.

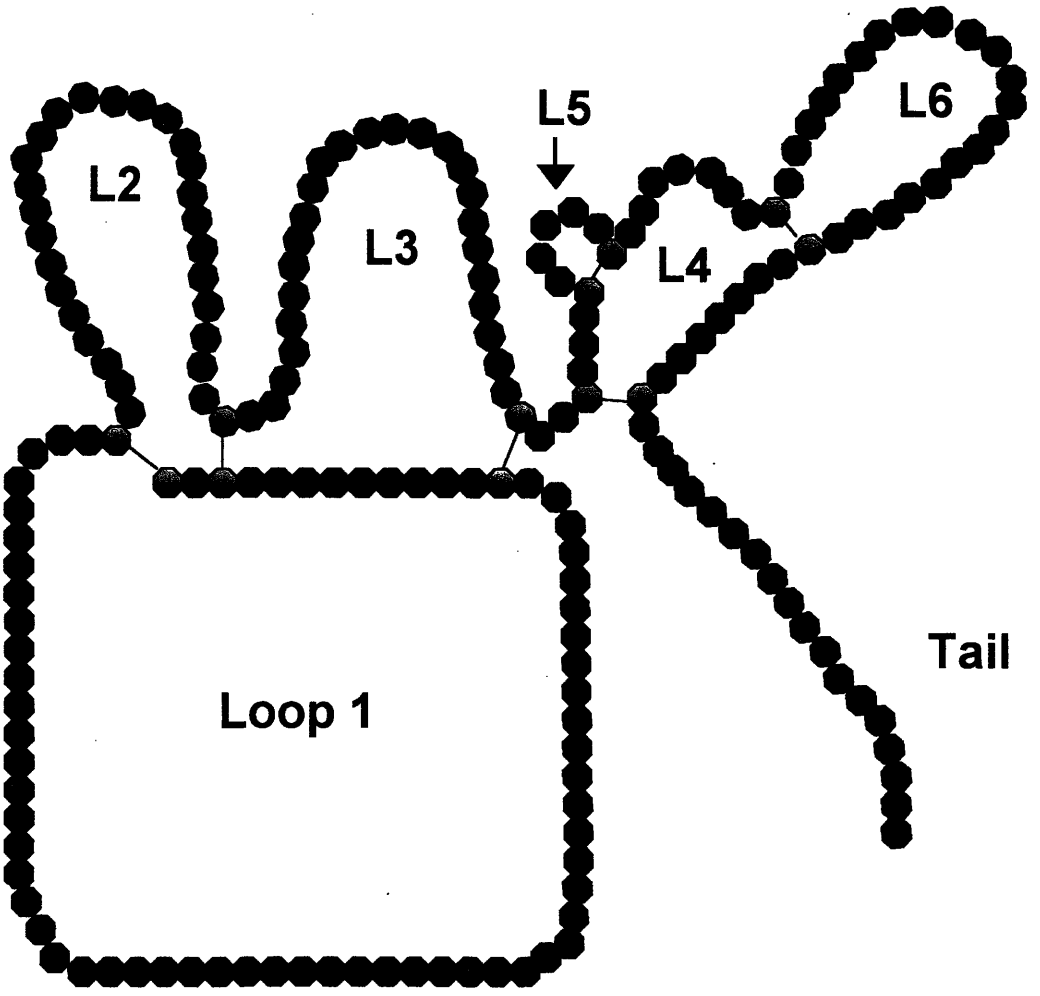


Figure 4. Primary structure of TIMPs. Twelve conserved cysteine residues are shown in orange and the six resulting disulfide bonds are indicated. The N-terminal domain of TIMPs is comprised of the first three disulfide-bonded loops while the C-terminal domain is comprised of the last three loops and the C-terminal tail.

Importantly, TIMPs have been shown to decrease the growth of tumors *in vivo* when cancer cell lines are transformed to overexpress TIMPs. For example, TIMP-2 overexpression results in the inhibition of hemangioma tumor growth in mice (Vergani et al., 2001). Based on studies such as this and on the initial findings that TIMPs could inhibit angiogenesis, a series of synthetic metalloproteinase inhibitors have been designed for use as potential therapeutic agents in the treatment of cancer and other angiogenic diseases.

Synthetic MMP inhibitors were originally designed to inhibit the enzyme by binding the active site in a similar manner to substrate binding. This first generation inhibitors contained a hydroxamic acid functional group to chelate the catalytic zinc ion (Gomis-Ruth et al., 1997). Although other zinc-binding groups, such as carboxylates and sulphydryls, have been tested, hydoxamates were shown to be the most effective MMP inhibitors, with kinetics similar to TIMPs (Brown and Giavazzi, 1995). The first generation hydroxamate inhibitors, such as batimastat (BB-94), had poor bioavailability, while second generation inhibitors, such as marimastat, were designed to be orally available. However, there is no specificity in the enzymes inhibited. The pursuit of more effective synthetic inhibitors has been fueled by their universal ability to inhibit tumor cell invasion *in vitro*, and these findings have, in turn, promoted the use of synthetic inhibitors as anti-metastatic agents. Although studies using hydroxamate inhibitors have shown, for example, reduced lung metastasis formation in mice (Chirivi et al., 1994), other studies have shown that the same inhibitor, BB-94, can promote liver metastasis (Kruger et al., 2001). Furthermore, synthetic MMP inhibitors have had disappointing results in clinical settings, where prolonged use has resulted in untoward side effects,

including musculoskeletal pain. Despite these setbacks, the usefulness of synthetic MMP inhibitors in clinical settings is still under investigation, either for alternative diseases or in combination therapies. The new generation of synthetics includes the design of MMP inhibitors, which inhibit certain MMPs selectively.

Historically, the potential therapeutic value of endogenous, as well as of synthetic inhibitors of metalloproteinases has been predicated on their ability to directly inhibit MMP activity.

Section D: TIMP-2

Since it was first demonstrated that TIMPs could inhibit angiogenesis, numerous studies have sought to characterize the specific angiogenic processes effected by MMP inhibitors (Moses et al., 1990; Moses and Langer, 1991; Murphy et al., 1993; Anand-Apte et al., 1997). It is now widely appreciated that although all three TIMPs (TIMP-1, -2, -3) tested to date inhibit MMP activity and the migration of endothelial cells stimulated by angiogenic mitogens, these inhibitors differ in their ability to regulate other angiogenic processes (Murphy et al., 1993; Anand-Apte et al., 1997; Moses, 1997). For example, TIMP-2 has the ability to inhibit the proliferation of capillary endothelial cells driven by angiogenic mitogens, while TIMP-1 has been reported to be a modest stimulator of capillary EC growth (Murphy et al., 1993; Hayakawa et al., 1992). Although TIMP-3 has recently been reported to inhibit the proliferation of genetically modified aortic endothelial cells that overexpress the VEGF receptor KDR (Qi et al., 2003), previous studies have reported that TIMP-3 actually had no effect on capillary endothelial cell proliferation (Anand-Apte et al., 1997). TIMP-4, the latest member of the TIMP family

to be cloned has yet to be rigorously tested. Moreover, neither Batimastat (BB-94), a synthetic MMP inhibitor with potent MMP-inhibitory activity, nor immunoneutralizing antibodies to MMP-2, inhibit capillary EC proliferation (Murphy et al., 1993). In fact, Batimastat has been shown to stimulate the outgrowth of capillary vessels, another process required for successful angiogenesis (Koolwijk et al., 2001). The effects of TIMPs on angiogenic processes, compiled from the data above, are summarized in Table III. It has been suggested that the pleiotropic effects that MMP inhibitors have on specific angiogenic processes, such as the inhibition of endothelial cell proliferation by TIMP-2, may be independent of their metalloproteinase inhibitory activity.

Early studies on the growth modulating effects of TIMP-2 sought to determine whether TIMP-2 could bind to the cell surface of various cell lines (Hayakawa et al., 1994; Emmert-Buck et al., 1995; Corcoran et al., 1996; Ko et al., 1997; Itoh et al., 1998). These studies confirmed the existence of one or two potential cell surface receptors depending on the cell type tested, but never isolated or characterized these binding entities. Several studies have since shown that TIMP-2 is involved in the cell surface activation of pro-MMP2 (Strongin et al., 1995; Zucker et al., 1998; Hernandez-Barrantes et al., 2000). In fact, a tri-molecular complex of MT1-MMP/TIMP-2/pro-MMP-2 is known to exist at the cell surface (Strongin et al., 1995; Zucker et al., 1998; Cao et al., 1998; Hernandez-Barrantes et al., 2000; Maquoi et al., 2000). In this complex, TIMP-2 acts as the "anchor" that recruits pro-MMP-2 to the cell surface where it can be subsequently activated by a second unbound MT1-MMP molecule.

Intense attention has focused on understanding the structural components of each of the proteins involved in the tri-molecular complex that are responsible for their

	MMP Activity	Capillary EC Migration	Capillary EC Proliferation
TIMP-1	↓	↓	↑
TIMP-2	↓	↓	↓
TIMP-3	↓	↓	No Effect
TIMP-4	↓	ND	ND

Table III. Summary of the known *in vitro* anti-angiogenic effects of TIMPs. All four TIMPs inhibit MMP activity. Of the TIMPs tested to date, TIMPs 1-3, all inhibit capillary endothelial cell migration but differ in their ability to inhibit capillary endothelial cell proliferation. TIMP-2 is the only TIMP that inhibits EC proliferation, while TIMP-1 is a modest stimulator of proliferation and TIMP-3 has no effect (references cited in text). The effect of TIMP-4 in these angiogenic processes has not been tested.

interactions. These studies have demonstrated that the N-terminal domain of TIMP-2 (T2N) binds to the catalytic site of MT1-MMP, while the C-terminal domain (T2C) binds to the C-terminal homeopexin (PEX) domain of pro-MMP-2 (Strongin et al., 1995; Zucker et al., 1998; Cao et al., 1998; Hernandez-Barrantes et al., 2000). A study in which the carboxy-terminal tail of TIMP-2 was replaced with the carboxy-terminal tail of TIMP-4 showed that the amino acid residues comprising the free C-terminal tail of TIMP-2 are required for cell surface activation of pro-MMP-2 (Kai et al., 2002). Based on these studies, it has been assumed that at least one of the cell surface interactions previously described is the result of the tri-molecular complex. However, this complex does not account for all of the binding interactions observed and may not be linked to the growth modulating effects of TIMP-2 (see discussion in Chapter 7).

A major goal of this thesis research was to identify and characterize the structural domains of TIMP-2 responsible for its endothelial cell growth-modulating activity and its anti-angiogenic activity *in vivo*.

Section E: TIMP-4

TIMP-4, the newest member of the tissue inhibitors of metalloproteinase family, was first cloned in 1996 (Greene et al., 1996). Interestingly, although many groups had attempted to isolate putative new TIMPs, TIMP-4 was not isolated as a protein but rather was identified using expressed sequence tag sequencing and homology searching. Not surprisingly, then, TIMP-4 expression was found to be either absent or very low in most tissues, with the exception of heart where TIMP-4 expression levels were increased. As with other TIMP family members, Ki values for the inhibition of MMPs was in the

nanomolar range (Liu et al., 1997; Stratmann et al., 2001). Structurally, TIMP-4 is most similar to TIMP-2, sharing approximately 50% sequence identity and 70% homology (Table IV). In fact, like TIMP-2, TIMP-4 has been shown to bind to the PEX domain of MMP-2 (Bigg et al., 1997). Although TIMP-4 has been shown to inhibit MT1-MMP, it does not promote pro-MMP-2 activation (Bigg et al., 2001) as TIMP-2 does.

To date, only twenty-five studies specifically focus on TIMP-4, including those described above. Most of the work on TIMP-4 has focused on its role in cardiac pathology based on the observations that TIMP-4 is primarily expressed in normal heart tissue (Greene et al., 1996) but that levels decrease after heart failure (Mujumdar and Tyagi, 1999). It has been suggested that perhaps the tissue specific expression of TIMP-4 in heart might explain why myocardial tumors are so rare. In a study of the in vitro effects of TIMP-4 on cardiac tumor cells, TIMP-4 was found to stimulate apoptosis of transformed cardiac fibroblasts, while not affecting the growth of normal fibroblasts (Tummalapalli et al., 2001). Additionally, TIMP-4 has been shown to inhibit platelet aggregation, suggesting that TIMP-4 is involved in the regulation of platelet aggregation and recruitment (Radomski et al., 2002). Despite the conclusions drawn from these results, not much is actually known about the normal function of TIMP-4 or its possible role in angiogenesis and tumor growth. What is known is that TIMP-4 decreases the migration and invasive potential of cancer cell lines *in vitro* (Wang et al., 1997; Liu et al., 1997; Celiker et al., 2001).

Breast cancer cell lines overexpressing TIMP-4 showed decrease invasive potential *in vitro* and decreased tumor growth *in vivo* (Wang et al., 1997), although TIMP-4 did not result in decreased metastasis formation. In fact, this study is the only

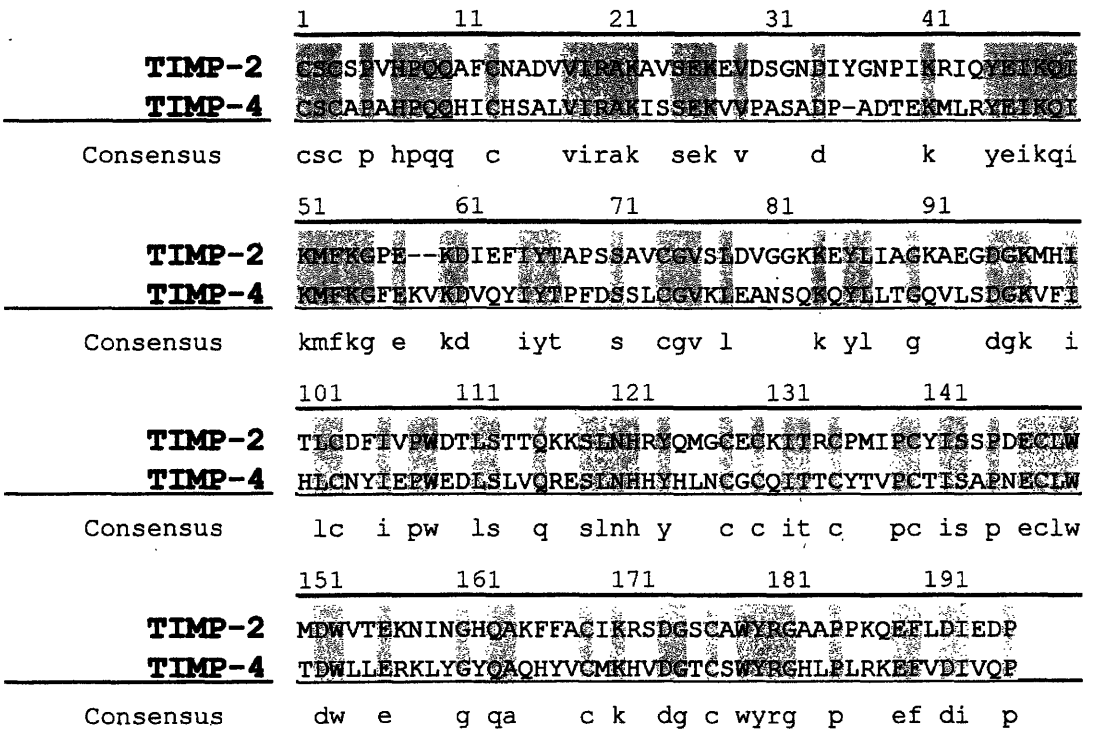


Table IV. Sequence alignment of TIMP-2 and TIMP-4. TIMP-4 shares approximately 50% sequence identity and 70% sequence homology with TIMP-2. Conserved residues are highlighted in yellow and identified below the sequence alignment.

one that has specifically attempted to determine the effects of TIMP-4 on angiogenesis, in that they observed decreased microvascular density in tumors overexpressing TIMP-4 when compared to control tumors, although the number of vessels was small (3.1 vessel/ field versus 5.7 vessels/ field). Interestingly, when the same tumors were treated with adenovirus-delivered TIMP-4, the tumors grew faster and had decreased apoptotic index (Jiang et al., 2001). The authors hypothesize that the local concentration of TIMP-4 probably dictates the net effect, with higher levels leading to inhibition while lower levels result in anti-apoptotic effects. However, in studies where TIMP-4 was delivered by intramuscular adenoviral transfection to mice bearing Wilm's tumors, tumor growth was inhibited (Celiker et al., 2001). Given the seemingly contradictory results on tumor growth, it is also possible that the conclusion that TIMP-4 inhibits angiogenesis based on microvascular density observations may also not be true in all cases.

Very little is known about the ability of TIMP-4 to directly affect angiogenesis. Although Ma and coworkers found that corneal wound healing resulted in higher levels of TIMP-4 being expressed, they did not test whether the opposite, whether TIMP-4 affects the neovascularization associated with wounding (Ma et al., 2003). It has largely been assumed that TIMP-4, as an MMP inhibitor, must inhibit angiogenesis, despite the contradictory results that TIMP-4 gene transfer has had in tumor growth and despite the fact that TIMP-4 has not been specifically tested. In fact, TIMP-4 protein has never been tested for any in vivo activity. Given the sequence homology of TIMP-4 to TIMP-2, and their shared ability to bind MMPs through their carboxy-terminal domains, we hypothesized that TIMP-4 would also inhibit the proliferation of endothelial cells in vitro

and angiogenesis in vivo. A major focus of this dissertation was to characterize the potential anti-angiogenic effects of TIMP-4.

Chapter 2: Uncoupling of the MMP-inhibitory and anti-proliferative activities of TIMP-2

Over the past decade it has largely been assumed that all TIMPs inhibit angiogenesis via the direct inhibition of MMP activity. This assumption is buttressed by the fact that TIMPs have been shown to inhibit the migration and invasion of various cell lines. However, more recent studies have pointed to the seemingly contradictory effects of TIMPs in tumor growth and angiogenesis and suggest that TIMPs might possess bi-phasic effects (Yan and Moses, 2001). These findings, along with the disappointing performance of synthetic MMP inhibitors in clinical trials (Coussens et al., 2002), further suggest that MMP inhibitors might not universally inhibit angiogenesis, as previously thought. In fact, in studies that specifically tested the ability of TIMPs to inhibit angiogenic processes, important differences were found. For example, although all the TIMPs tested to date (TIMP-1, -2, -3) can inhibit capillary endothelial cell migration, they differ in their ability to inhibit capillary endothelial cell proliferation. Of particular interest is the fact that TIMP-2 is the only TIMP that has been consistently shown to be a potent inhibitor of capillary endothelial cell proliferation (Murphy et al., 1993). In contrast, TIMP-1 has been shown to be a modest stimulator of capillary endothelial cell proliferation (Hayakawa et al., 1992), while TIMP-3 had no significant effect (Anand-Apte et al., 1997).

Since all TIMPs are capable of inhibiting matrix metalloproteinases equally well and since the anti-metalloproteinase activity has been demonstrated to reside in the N-terminal portion of the TIMP molecules (Murphy et al., 1991; O'Shea et al., 1992; Butler

et al., 1999; Fernandez-Catalan et al., 1998; Muskett et al., 1998), it was hypothesized that the unique anti-proliferative activity of TIMP-2 is housed in the more variable C-terminal domain of the molecule, and constitutes a second anti-angiogenic function of this TIMP, independent of MMP-inhibitory activity. In order to support this hypothesis a series of structure-function studies were conducted to identify the structural determinants of the anti-angiogenic activity of TIMP-2.

Section A: Cloning of Human TIMP-2 and TIMP-2 Domains

Expression System

Choosing an expression system in which to produce TIMP-2 and the TIMP-2 fragments was largely based on the need for proper three-dimensional folding of the proteins. Although E. coli systems result in higher yields than other systems, one of the disadvantages is that most proteins form inclusion bodies that then must be treated with strong reducing agents and the proteins subsequently refolded. Correct refolding is assessed using an assay to a known bioactivity of the protein. Although the ability of TIMP-2, and even T2N, to inhibit MMP activity could be tested, the only bioassay available for T2C would be an endothelial cell proliferation assay. However, since this is the hypothesis being tested, a eukaryotic system was more desirable in that eukaryotic systems would be most likely to produce correctly processed proteins.

The methylophilic yeast expression system, *Pichia pastoris*, was chosen because it has been successfully used to express disulfide-bonded proteins at higher yields than mammalian cell systems (Ikegaya et al., 1997; Sun et al., 1997), while still providing a eukaryotic environment in which correct protein processing and folding can occur.

Although mammalian systems have previously been used to produce other TIMPs, the yields are typically low. Furthermore, since expressed proteins are secreted into media typically containing serum, purification is often more complicated than in yeast systems where the growth media contains very few other proteins. A third complication of using mammalian systems is the possibility of contamination of protein preparations with other interacting proteins. For example, because MMP-2 has been shown to bind TIMP-2, TIMP-2 expressed in mammalian systems is often contaminated with small amounts of MMP-2 (Stetler-Stevenson et al., 1989). The *Pichia pastoris* system has the additional advantage of utilizing the same tools used to grow and transform bacteria, such that no special equipment is required for expression. The pPICZαA yeast expression vector contains a Zeocin resistance marker for selection both in yeast and in *E. coli*, and a c-terminal His-tag to facilitate protein purification. A portion of the gene encoding for the yeast alcohol dehydrogenase (AOX1) is included to provide a site for recombination into the yeast genome, as well as placing the gene of interest under the control of the inducible AOX1 promoter.

Cloning Strategy

Human TIMP-2 was cloned from a human heart cDNA library using hi-fidelity PCR and primers designed to produce full-length *TIMP-2,* a 3'-deletion fragment and a 5'-deletion fragment (Figure 5A). Cloning of these gene fragments result in the expression of two domains of TIMP-2 as distinct proteins. The first is composed of the three N-terminal disulfide-bonded loops (~15 kD) designated T2N, and the second is composed of the three C-terminal disulfide-bonded loops (~8.5 kD) designated T2C. In addition, a mutant

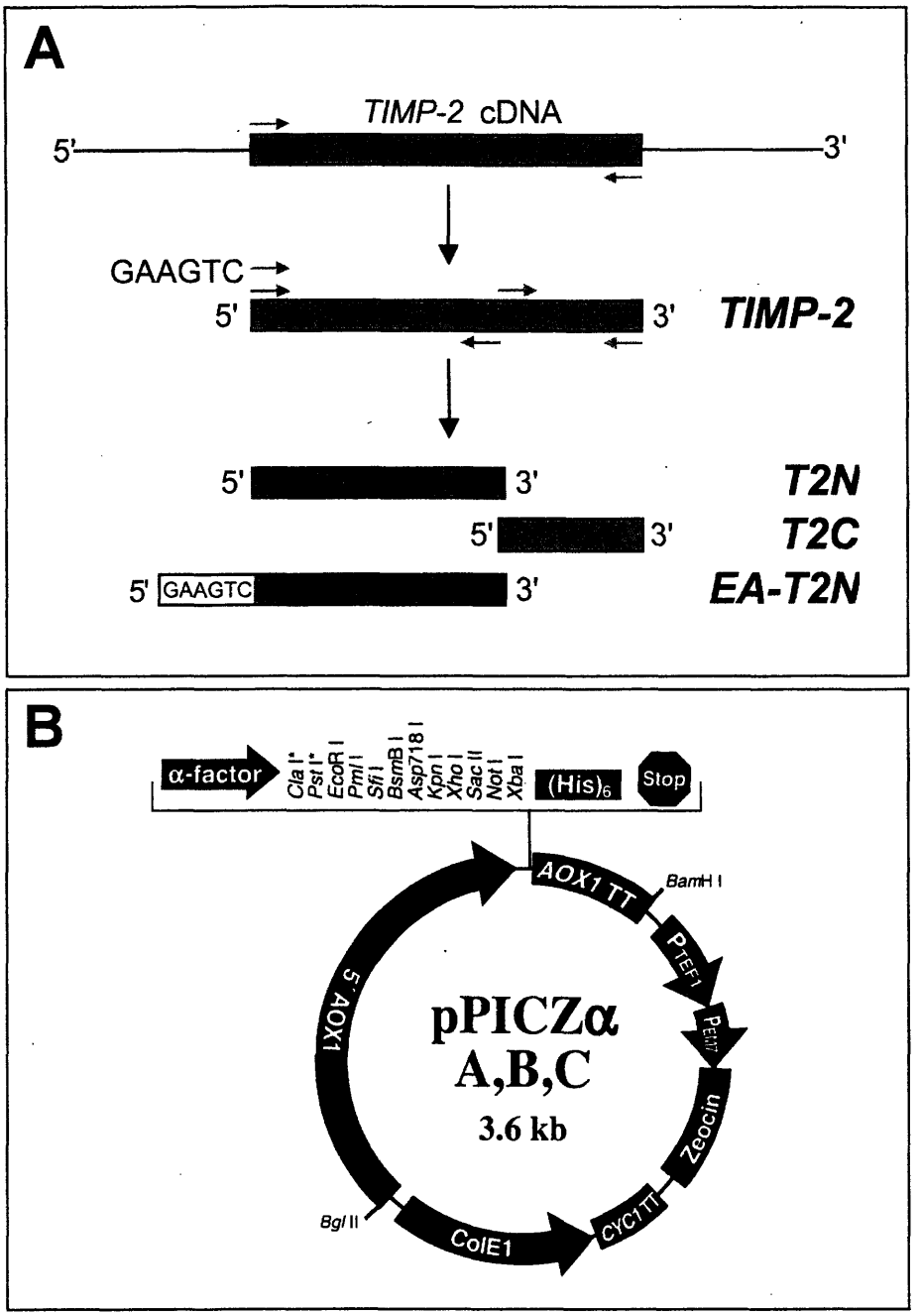


Figure 5. Cloning strategy. (A) *TIMP-2* was cloned from a human heart cDNA library using high-fidelity PCR with specific primers to produce the mature form of TIMP-2. Subsequent PCR was performed to yield two *TIMP-2* truncation products encoding for the N-terminus (T2N) and the C-terminus (T2C) of TIMP-2 as well as a PCR product encoding a mutant form of T2N, EA-T2N. (B) PCR products were digested with Xho I and Xba I and cloned into the pPICZαA *Pichia pastoris* expression vector.

form of *T2N* that encodes for a form of T2N with additional glutamine and alanine residues at the N-terminus of the protein, and designated EA-T2N, was designed and expressed for use as a control for MMP-inhibition. PCR products were also designed to carry an Xho I restriction enzyme cleavage site on the 5'-end, and an Xba I cleavage site on the 3'-end.

PCR products were digested overnight with both Xho I and Xba I, ligated into pPICZαA (Figure 5B) and transformed into Top10 *E. coli* for plasmid amplification. Candidate *E. coli* clones were sequenced to verify gene sequence integrity and correct frame insertion. Verified clones of each gene were linearized at the AOX1 gene and transformed into X-33 and GS115 strains of *P. pastoris* by electroporation. Linearized pPICZαA was also transformed to provide a vector control for protein expression. Positive clones were selected for over the next 36 hours on plates containing 100 μg/ml Zeocin and then re-streaked to obtain single colonies. Recombination of the TIMP-2 and TIMP-2 fragments into the yeast genome was verified by PCR analysis of the genomic DNA using genomic primers to yield 1130bp, 925bp, and 750bp products for intact *TIMP-2, T2N* and *T2C*, respectively (Figure 6).

Section B: Expression and purification of TIMP-2 and TIMP-2 domains

Initial expression was verified for one clone of each gene for each of the two *Pichia* strains tested, X-33 and GS115. Surprisingly, no proteins were detected in the media of any of clones from the GS115 strain, while both TIMP-2 and T2N expressed in X-33 showed detectable expression levels by silver-stained SDS-PAGE. T2C expression was

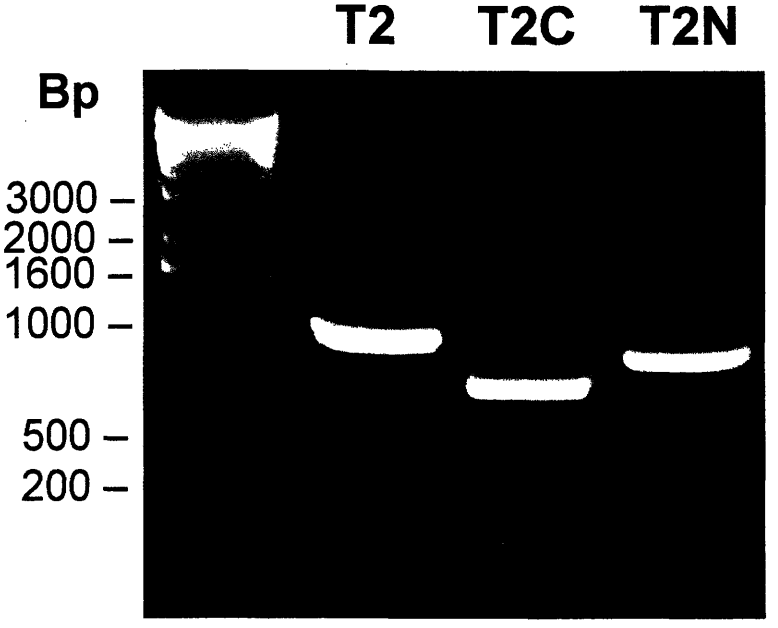


Figure 6. ***TIMP-2*** **and** ***TIMP-2*** **truncation products in** ***Pichia pastoris.*** PCR products cloned into the pPICZαA vector were transformed into X-33 wild-type *Pichia.* Incorporation of the genes of interest into the yeast genome by homologous recombination at the AOX1 site was verified by PCR using yeast-specific primers.

only detectable by western analysis for this particular clone. Four X-33 clones of each of the proteins to be tested were then tested, and the ones with the highest expression level of each protein were chosen for future experiments. Protein identities were verified by N-terminal sequencing.

Before going forward, a considerable amount of effort was spent optimizing the expression conditions for each of the proteins being produced. In the course of these studies the following approaches were evaluated: expression in minimal media versus rich media, buffered versus un-buffered media, variation of cell density at induction time, and length of time post-induction for protein harvest. Results of these experiments are as follows: (1) Although minimal media yields a cleaner protein prep, the expression levels were lower, such that proteins could only be detected by western blotting. (2) Buffered mediaresulted in increased protein yields, presumably by preventing protein degradation during expression. (3) Optimal expression was achieved when ell density at time of induction was 5 X 10^6, or early log phase. (4) Time course experiments in which protein yield was tested at 24, 48, 72, and 96 hours post-induction showed no marked increase in yield past 48 hours for most proteins and 24 hours in the case of T2C. In summary, optimal expression conditions determined from these experiments were as follows: 25 ml overnight cultures were grown at 30° C in BMGY media (2% peptone, 1% yeast extract, 100 mM potassium phosphate pH 6.0, 1.34% yeast nitrogenous base, 1% glucose) containing 100μg/ml Zeocin and cell pellets were collected the next day by centrifugation at 1500g. Cultures were induced by re-suspending the cell pellets in 250 ml of methanol-

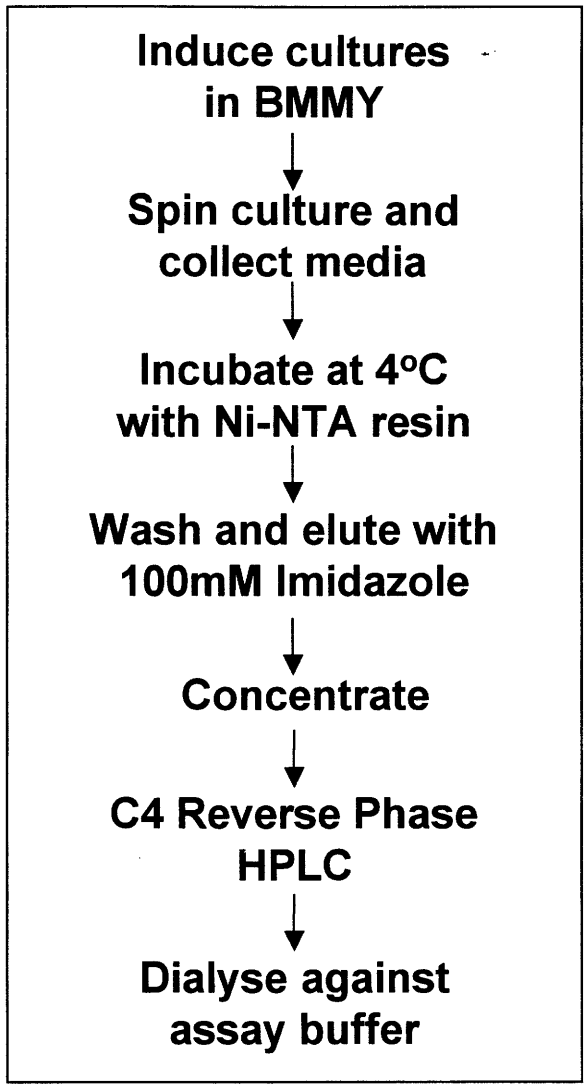


Figure 7. Protein purification strategy

containing media (BMMY: 2% peptone, 1% yeast extract, 1% cassamino acids, 100 mM potassium phosphate pH 6.0, 1.34% yeast nitrogenous base, 1% methanol), and allowed to grow for 24- 48 hours, depending on the protein being expressed. The addition of 1% cassamino acids to the induction media was found to reduce the degradation of the expressed proteins prior to harvesting. Media containing the secreted expressed protein was cleared of cell content by centrifugation.

A major challenge in the course of these studies involved designing a purification scheme for all expressed proteins which would maximize the yields of the pure proteins while reducing the amount of time involved and thus the possibility of protein degradation during purification. An optimal purification scheme was developed as shown in Figure 7. After media collection, an initial step of histidine-affinity chromatography was performed. Expressed proteins with incorporated C-terminal His-tags were allowed to bind a Ni-NTA resin in batches at 4° C for 1 hour. The resin was then loaded unto a glass column, washed with phosphate buffer and eluted in buffer containing 100 mM Imidazole. Elutes were concentrated using a 3,500 MWCO centrifugal concentrators and then subjected to a second round of purification using reverse phase HPLC. Sample chromatograms of the purification of TIMP-2 and the TIMP-2 domains, T2N and T2C, as well as the mutant EA-T2N, by reverse phase HPLC are shown in Figure 8. Each batch of protein was divided into three HPLC runs, which were then pooled, dialyzed versus water and lyophilized overnight. Sample purity was monitored by SDS-PAGE followed by silver-staining as described in the Methods section

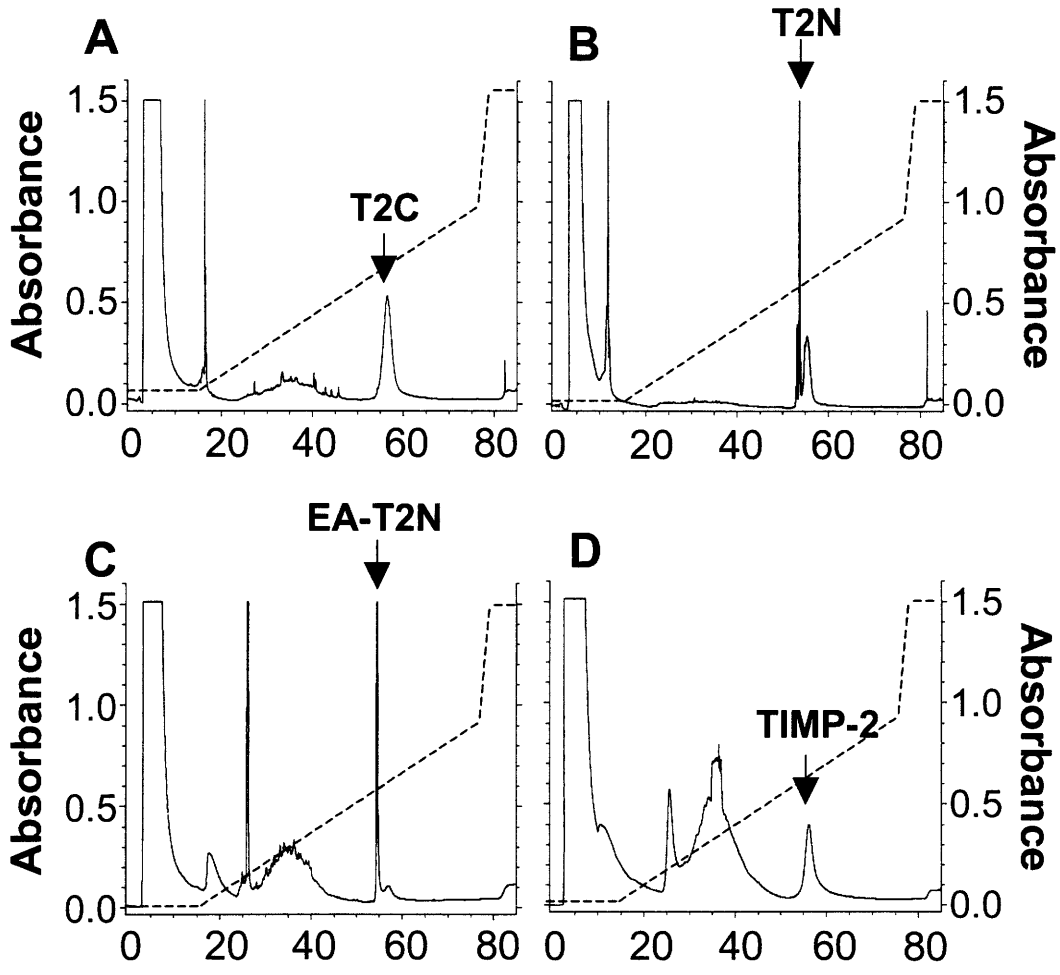


Figure 8. C4 reverse phase HPLC purification of TIMP-2 and TIMP-2 domains. Representative chromatograms of the purification of each of the expressed proteins (indicated by arrows) in the course of these experiments are shown.

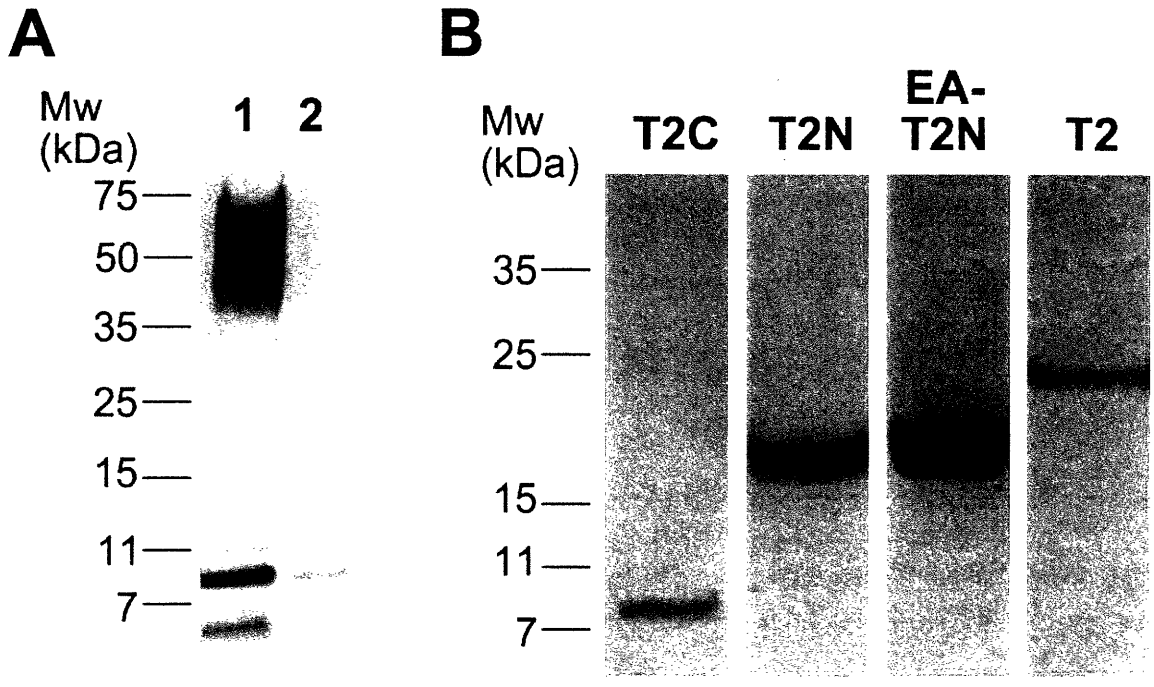


Figure 9. Purification and yield of TIMP-2 and TIMP-2 domains. Protein purification was monitored by SDS-PAGE followed by silver staining. An example of a silver-stained SDS-PAGE gel of the His-affinity purified HPLC starting material of T2C (Lane 1) and the purified T2C (Lane 2) after reverse phase HPLC is shown in panel (A). (B) This representative silver-stained SDS-PAGE gel of each of the expressed TIMP-2 and TIMP-2 domains illustrates the purity and relative yield of each of the expressed proteins.

of this dissertation. Figure 9A shows a sample silver-stained gel of the T2C HPLC sample starting material after His-affinity (Lane A) and once purified by reverse phase HPLC (Lane B). Other techniques tested included size exclusion and ion exchange chromatography as second purification steps. However, the success of both of these strategies is dependent on the composition and volume of the starting material, which meant that additional dialysis and concentration steps were required prior to chromatography. This resulted in increased the purification time, as compared to reverse phase chromatography. Additionally, removal of salts after purification and prior to lyophilizing, required extensive dialysis in water with two exchanges, which resulted in increased protein degradation. In contrast, protein eluted from the reverse phase column could be dialysed in one quick step without exchanges to successfully remove acetonitrile prior to lyophilization. Once purified to homogeneity, identity of each protein was verified via N-terminal amino acid sequencing. Figure 9B shows a representative silver-stained gel of each of the expressed proteins. Typical yields of each sample protein were as follows: 1.2 mg/L for T2N, 240 μg/L for T2C, 15 mg/L for EA-T2N, and 300 μg/L for intact TIMP-2.

Section C: Inhibition of MMP activity by T2N

To demonstrate that the N-terminal domain of TIMP-2, T2N, retains MMP-inhibitory activity as previously reported (Murphy et al., 1991; O'Shea et al., 1992; Tolley et al., 1993; Fernandez-Catalan et al., 1998; Muskett et al., 1998; Butler et al., 1999) and to validate the ability of this expression system to produce biologically active protein, we

tested TIMP-2, the TIMP-2 domains, and an MMP-inhibition deficient form of T2N, EA-T2N, for their ability to inhibit MMP activity using a standard quantitative radiometric MMP assay (Moses et al., 1990).

For this assay, type I collagen was labeled with ^{14}C using an acetylation reaction as described in the Methods section of this dissertation. The ^{14}C-labeled collagen was added to 96 well plates and allowed to polymerize. Metalloproteinase activity is determined from the amount of ^{14}C released via collagen degradation. To determine inhibitory activity, wells were treated with a known amount of type I collagenase plus test sample or with collagenase alone. Type I collagenase was prepared from explant cultures of excised bovine corneas as described in the Methods section. Plates containing 200 µl/well of either the corneal type I collagenase plus sample or the collagenase alone were incubated at 37° C for 2.5 hours to allow for release of ^{14}C by the enzyme. Supernantants were then analyzed in a scintillation counter and percent inhibition of collagenolytic activity was calculated. An IC_{50} was defined as the amount of protein necessary to inhibit the proteolytic activity of collagenase by 50%. As expected, T2N inhibited MMP activity at concentrations comparable to intact TIMP-2, with an IC_{50} of approximately 7.5 nM (Figure 10). No significant inhibition of MMP activity was detected for either T2C or EA-T2N, even at concentrations approximately 100-fold higher than that of the IC_{50} of T2N (Figure 10).

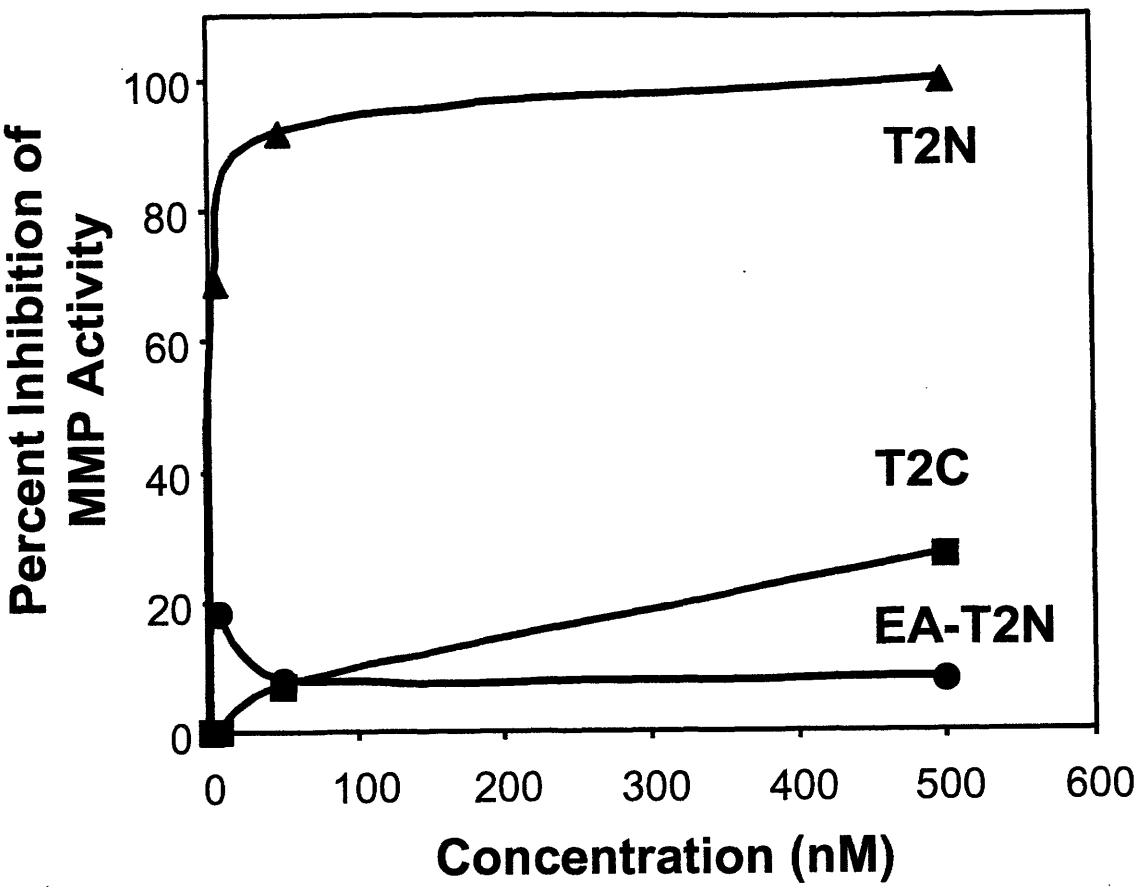


Figure 10. T2N retains MMP-inhibitory activity. The expressed TIMP-2 domains were tested for their ability to inhibit MMP activity using a radioactive collagen film assay. T2N inhibited MMP activity with an IC_{50} of approximately 5 nM, which is consistent with the inhibition observed with intact TIMP-2. Neither T2C nor the mutant EA-T2N significantly inhibited MMP activity even at doses 100-fold higher.

Section D: T2C, but not T2N, inhibits the proliferation of normal capillary endothelial cells

Since the MMP-inhibitory activity of TIMPs has been attributed to the highly conserved N-terminal portion of the molecules, we hypothesized that the anti-proliferative effect of TIMP-2 on capillary EC resides in the more variable C-terminal domain (Table II). We therefore tested individual TIMP-2 domains for their ability to suppress capillary EC proliferation *in vitro.*

Briefly, capillary EC were plated on pre-gelatinized 96 well plated at a density of 2,000 cells per well in DMEM supplemented with 5% calf serum and allowed to attach for 24 hours. The next day, cells were treated with fresh media with or without 1ng/ml bFGF and challenged with the test proteins at various concentrations. All samples were tested in duplicate or triplicate. Control wells contained cell treated with media alone or media with bFGF. After 72 hours, the media was removed and the cells were lysed in buffer containing Triton X-100 and the phosphatase substrate *p*-nitrophenyl phosphate. After a two-hour incubation at 37°C, NaOH was added to each well to terminate the reaction. Cell density was determined by colorimetric analysis using a multiwell plate reader, and percent inhibition of bFGF-driven proliferation calculated. All activities were verified by cell counting using a Coulter Counter.

T2C alone, inhibited capillary EC proliferation driven by the angiogenic stimulant basic fibroblast growth factor (bFGF) with an IC_{50} of approximately 140 nM (Figure 11) and showed no evidence of cytotoxicity. These results are consistent with those previously reported for TIMP-2 (Murphy et al., 1993). Although T2N is responsible for

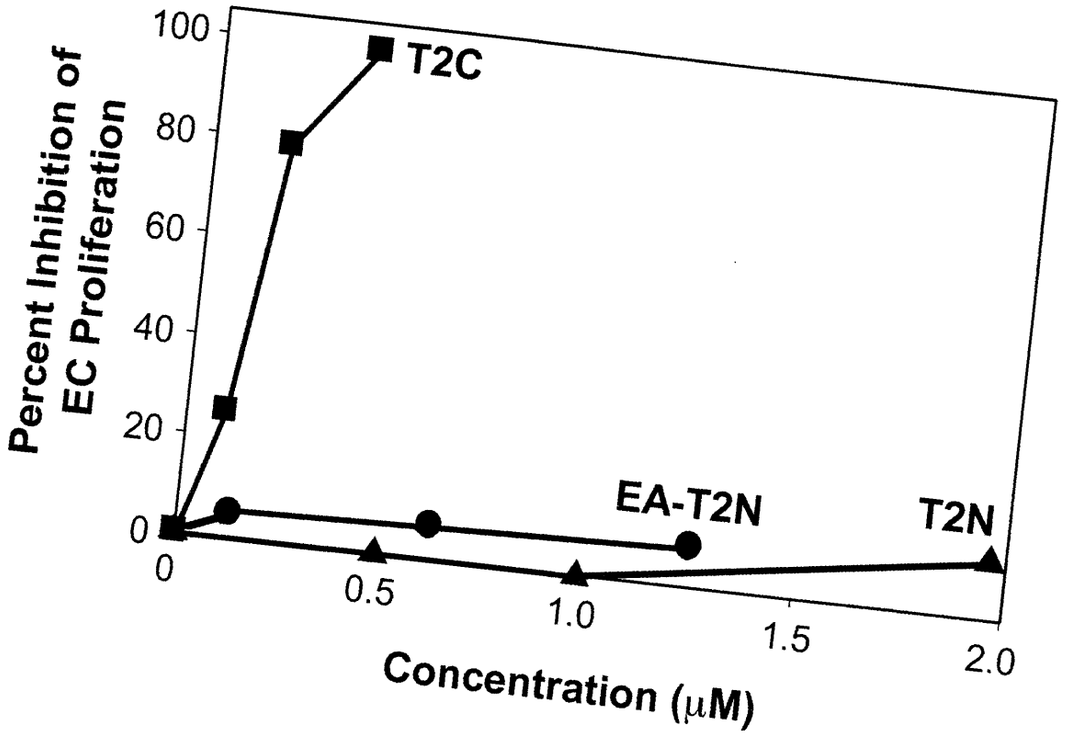


Figure 11. T2C inhibits capillary endothelial cell proliferation. The TIMP-2 domains were next tested for their ability to inhibit capillary EC proliferation. T2C inhibited bFGF-stimulated capillary EC proliferation at doses comparable to those previously reported for TIMP-2, while neither T2N nor the mutant EA-T2N had any significant activity in this assay.

inhibition of MMP activity, it had no significant effect on capillary EC proliferation. These results demonstrate that the inhibitory effect of TIMP-2 on capillary EC proliferation is independent of MMP inhibition, and that these bioactivities can be dissociated from each other. This unique anti-proliferative activity of T2C represents a second anti-angiogenic domain within TIMP-2.

Section E: Summary

We have expressed the N-terminal and C-terminal domains in TIMP-2 as two independent proteins and have tested them for their ability to inhibit MMP activity and capillary endothelial cell proliferation. Consistent with previous studies, we found that the N-terminal domain, T2N, retains MMP-inhibitory activity. Neither the C-terminal domain, T2C, nor the mutant form of T2N, EA-T2N, had any significant MMP-inhibitory activity. In contrast, T2C inhibited capillary endothelial cell proliferation, while T2N had no significant effect. These results demonstrate that the anti-proliferative activity of TIMP-2 is housed in the C-terminal domain. We have succeeded in uncoupling the anti-proliferative and MMP-inhibitory activities of TIMP-2 and have shown that these two activities can exist independently of each other.

Chapter 3: *In vivo* anti-angiogenic activity of TIMP-2 domains

Given that MMP inhibitors have been shown to inhibit angiogenesis and that T2C independently inhibits capillary endothelial cell proliferation, we next examined whether these two domains, which inhibit angiogenic processes *in vitro*, could also inhibit angiogenesis *in vivo* in two separate bioassays. The chick chorioallantoic membrane assay (CAM) is the most accepted *in vivo* test to examine the effects of angiogenic modulators on the growing vascular network of the developing chick, and is commonly used as a first-pass screen for potential anti-angiogenic agents. The mouse corneal pocket assay is a more complex *in vivo* system in which angiogenesis in the avascular cornea is driven by the exogenous addition of an angiogenic mitogen most often via the release of mitogen from an implanted sustained release polymer pellet. This system examines the effects of a potential anti-angiogenic agent in the context of aberrant angiogenesis in an adult, where an imbalance in favor of a positive regulator of angiogenesis results in an abnormal event, corneal neovascularization.

Section A: TIMP-2 domains independently inhibit angiogenesis *in vivo* in the chick chorioallantoic membrane assay (CAM)

T2C and T2N were first tested for their ability to inhibit embryonic, unstimulated angiogenesis *in vivo* in the chick chorioallantoic membrane assay (CAM). For these assays, three-day old chick embryos were removed from their shells and incubated in plastic Petri dishes for three days. On the sixth day, samples and controls mixed into methylcellulose discs were applied to the surfaces of developing CAMs, above the dense

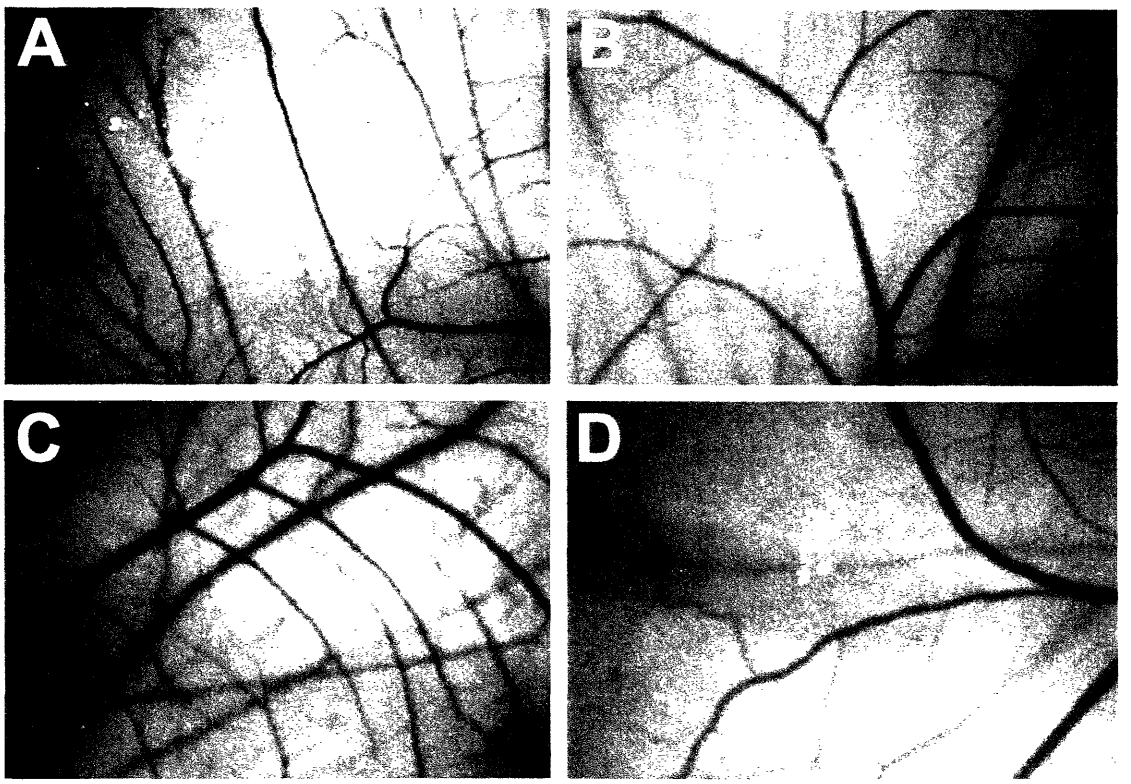


Figure 12. TIMP-2 and TIMP-2 domains suppress embryonic angiogenesis in the chick chorioallantoic membrane assay (CAM). Representative CAMs in which equivalent doses of each of the expressed proteins were tested are shown. (A) T2C inhibited neovascularization and resulted in large avascular zones as well as apparent vessel regression, characterized by the tortuous appearance of the remaining vessels. (B) T2N also inhibited embryonic neovascularization in the CAM with smaller avascular zones and was characterized by the apparent dissolution of the vessels. The MMP-inhibition deficient EA-T2N did not inhibit in the CAM (C). Intact TIMP-2 also resulted in avascular zones which were reminiscent of the CAMs treated with T2N (D).

subectodermal plexus. After 48 hours of incubation, the eggs were examined for vascular reactions under a dissecting scope (60X) and photographed. All determinations were made by 3 independent laboratory members, in a double-blinded fashion. Approximately 120 eggs were tested in these experiments.

The addition of T2C or T2N to the chorioallantoic membrane resulted in a decrease in neovascularization. T2C produced avascular zones at doses as low as 112 pmol per egg in 64% of the CAMs tested, while T2N inhibited at doses approximately 5 fold higher in approximately 70% of the CAMs tested. EA-T2N, which is deficient in MMP- and capillary EC-inhibitory activity, did not result in significant inhibition of neovascularization (15%). TIMP-2 was used as a control. The representative CAMs shown in Figure 12 were treated with equivalent amounts of each of the proteins tested at the lowest dose in which inhibition should be achieved for T2N.

Of particular interest is the fact that appearance of the vasculature in CAMs treated with T2C or T2N differed significantly. CAMs treated with T2C had much larger avascular zones and the few remaining vessels had a tortuous appearance reminiscent of vessels undergoing regression (Burt et al., 1995). The inhibition of neovascularization in the vicinity of the methylcellulose disc containing T2N, however, could be characterized by a dissolution of CAM vessels. Given that numerous studies have demonstrated the requirement for increased MMP activity at the migrating edge of growing vessels during angiogenesis (Monsky et al., 1993; Partridge et al., 1997; Giannelli et al., 1997; Werb, 1997), the appearance of the CAMs treated with T2N is consistent with the direct inhibition of MMPs. The results with T2N are also consistent with those observed for CAMs treated with intact TIMP-2.

Section B: T2C inhibits bFGF-driven neovascularization in the mouse corneal pocket assay

The ability of the TIMP-2 domains to inhibit angiogenesis was next assessed in the mouse corneal pocket assay, where neovascularization was directly stimulated by the addition of an exogenous angiogenic mitogen. Hydron pellets containing sucrose octasulfate and, either test sample (5 μg) plus bFGF (40 ng/ml) or bFGF (40 ng/ml) alone, were prepared as follows: 500 μg of lyophilized protein was resuspended in 10 μl of sodium citrate buffer (20 mM NaCi, 1mM EDTA pH 5, 9% sucrose) and mixed with 4 μg of bFGF in 10 μl of sodium citrate buffer. Alternatively, 4 μg of bFGF were diluted in 20 μl of buffer to make control pellets. Protein/ bFGF mixes or bFGF alone were then added to 10 mg of sucrose octasulfate, vortexed and allowed to dry for 4 minutes in a speed vac. The dried mixture was resuspended with 10 μl of Poly(2-hydroxyethyl methacrylate) (Hydron) and spread evenly over a 10 X 10 grid on a Nytex nylon membrane and allowed to dry overnight. The next morning, the nylon membrane was carefully dissected to liberate 100 hydron pellets.

Pellets were implanted into corneal micropockets of C57Bl/6 mice as previously described (Moses et al., 1999). Briefly, six mice per treatment group per experiment were anaesthetized and the eyeball proptosed to facilitate micropocket formation. A single surface incision was made in the cornea below the pupil and a micropocket manufactured using the end of a small spatula. Hydron pellets were then inserted into the pocket and antibiotic ointment was applied to the eye to prevent infection. Each animal carried a pellet containing the test sample plus bFGF in one eye, and a control bFGF

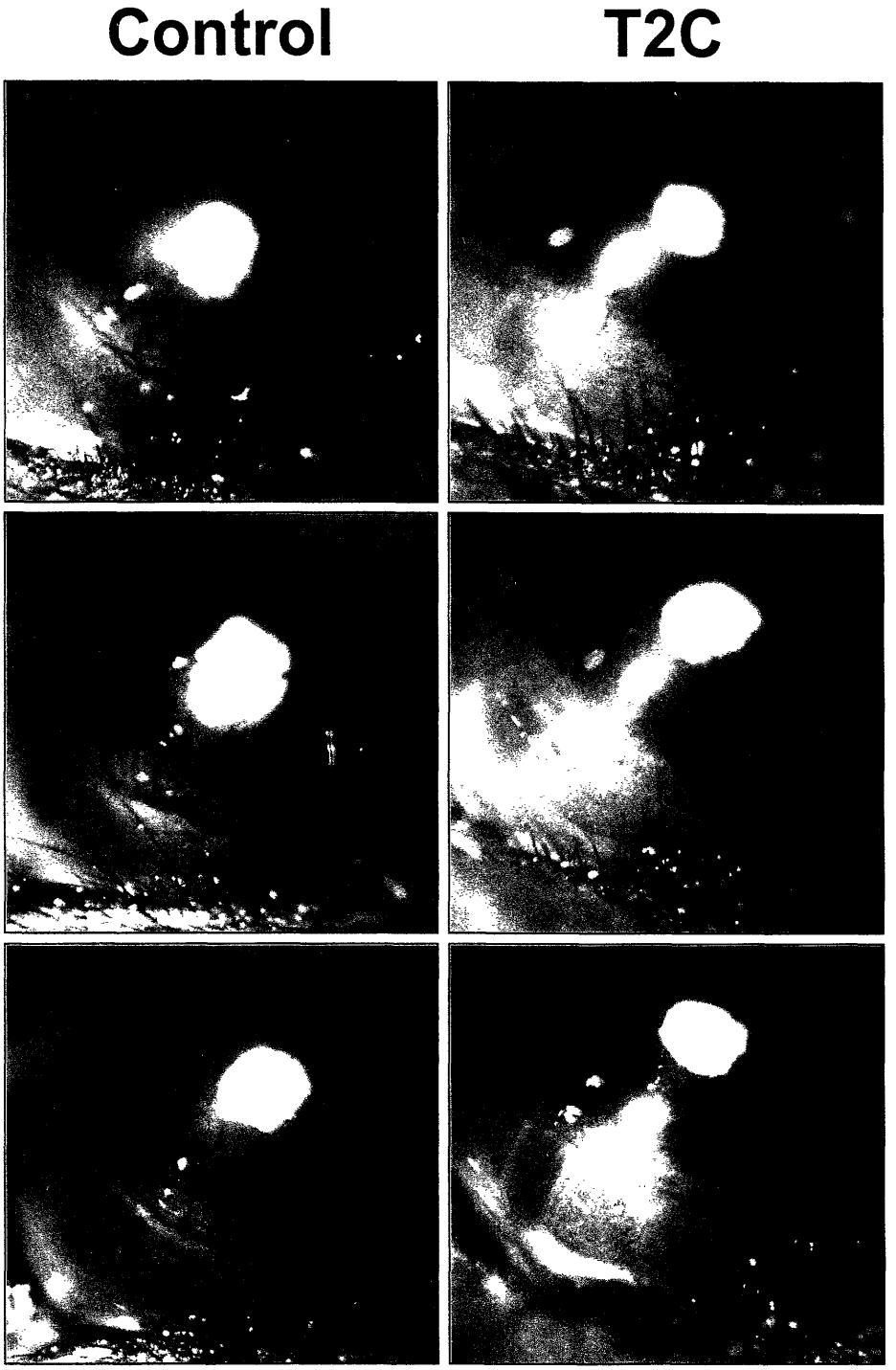


Figure 13. T2C inhibits mitogen-stimulated neovascularization *in vivo* in the mouse corneal pocket assay. The ability of the TIMP-2 domains to inhibit angiogenesis stimulated by the exogenous addition of an angiogenic mitogen (bFGF) in a polymer pellet was tested in the mouse corneal pocket assay. Representative corneas from three mice treated with T2C are shown on the right hand column. The control corneas for each of the mice shown are on the left column. T2C resulted in 87% inhibition of corneal neovascularization.

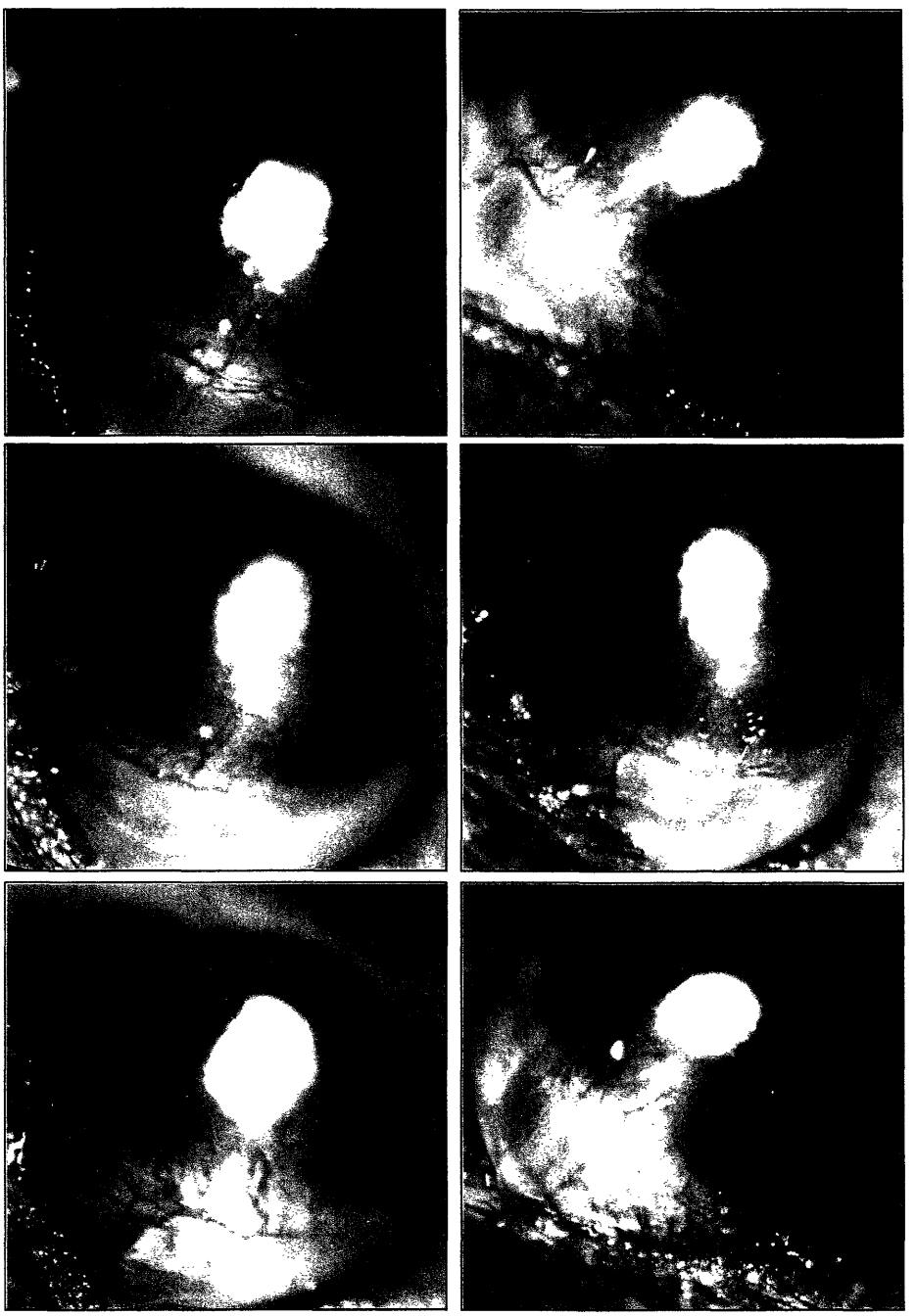


Figure 14. T2N modestly inhibited corneal neovascularization. Representative corneas of three mice treated with T2N are shown on the right hand column. Control corneas for each mice are shown on the column on the left. T2N resulted in only modest (37%) inhibition of corneal neovascularization.

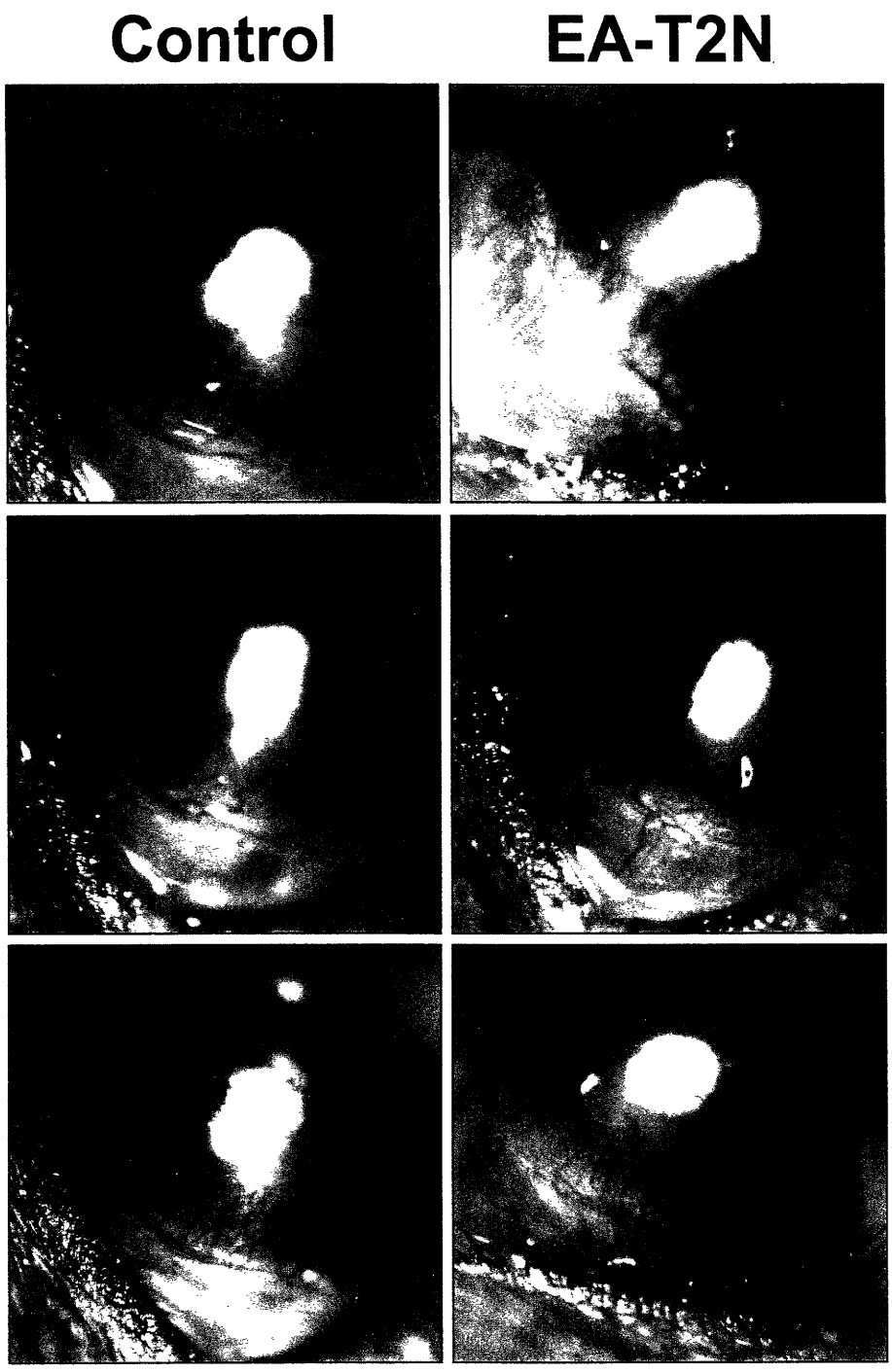


Figure 15. EA-T2N did not significantly inhibit corneal neovascularization. Representative corneas of three mice treated with EA-T2N are shown on the right hand column. Control corneas for each mice are shown on the column on the left. EA-T2N did not significantly inhibit corneal neovascularization. Importantly, the effect observed was not statistically different than those observed in corneas treated with T2N (see Figure 17).

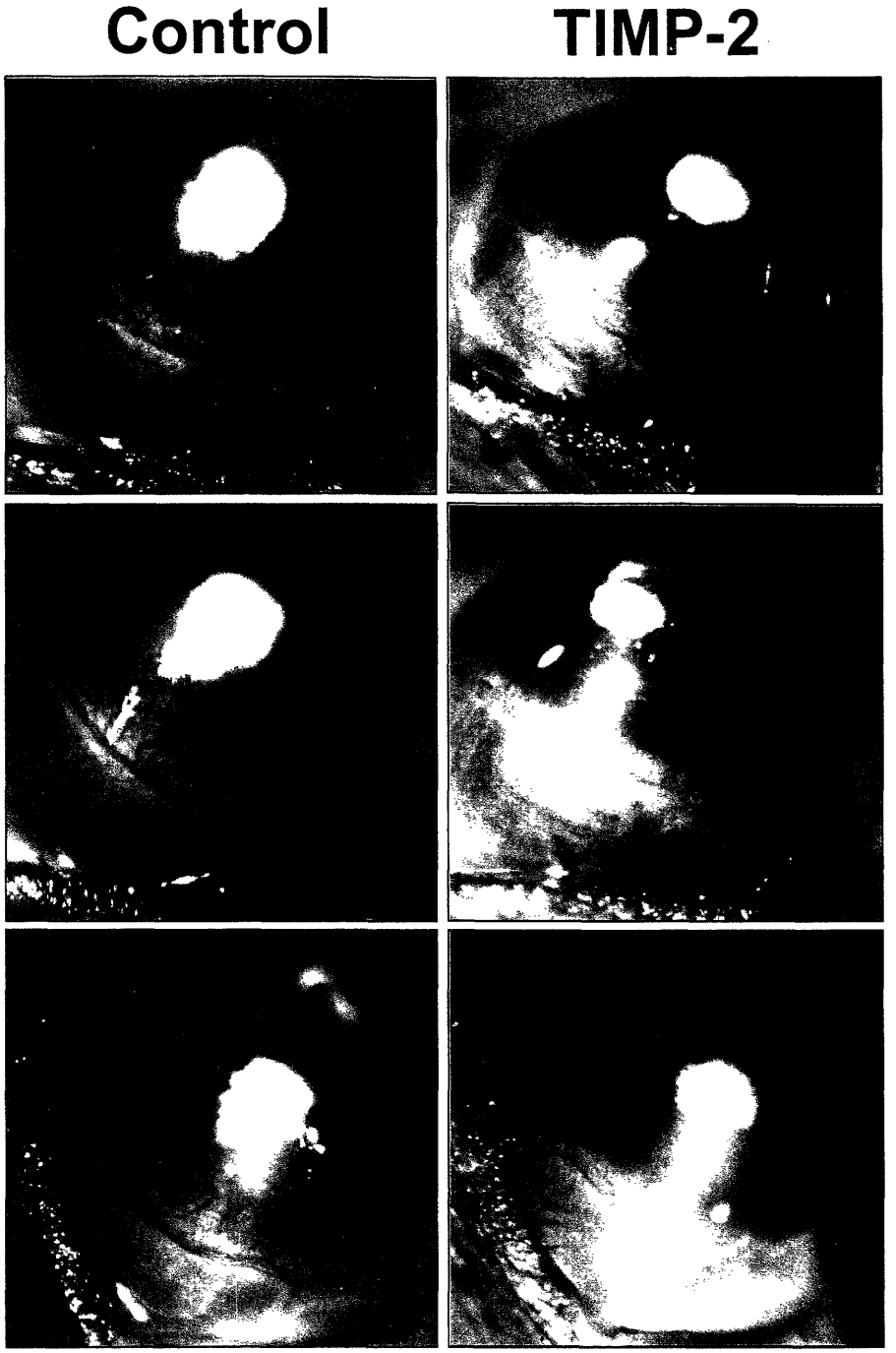


Figure 16. TIMP-2 potently inhibited corneal neovascularization. Representative corneas from mice treated with TIMP-2 are shown in the right column. Control corneas for each of the mice shown are on the left hand column. TIMP-2 resulted in the potent inhibition of mitogen-stimulated angiogenesis in the cornea which was statistically the same as those obtained in corneas treated with T2C.

pellet in the contralateral eye. After six days, angiogenesis was evaluated using a slit lamp microscope, and each eye was photographed. The area of neovascularization for each cornea was calculated from the length of the vessels invading the cornea (VL) as well as the clock hours (CH) covered, and percent inhibition was calculated using the standard quantitative formula VL x CH x 0.68 in control versus treated eyes for each animal.

T2C resulted in an 87% reduction of bFGF-driven corneal neovascularization when compared to the contralateral control eye treated with only bFGF (Figure 13). Surprisingly, treatment of the corneas with T2N resulted in only modest (37%) inhibition of angiogenesis (Figure 14) and did not differ significantly from the inhibition observed when corneas were treated with the MMP-inhibition deficient form of T2N, EA-T2N (Figure 15), suggesting that direct inhibition of MMP activity may not be sufficient to inhibit mitogen-driven angiogenesis. As expected, intact TIMP-2 resulted in potent inhibition of corneal neovascularization (Figure 16) and which was not statistically significant different from those obtained with T2C. The data represents the average percent inhibition obtained from separate experiments of 18 corneas per treatment group. Interestingly, T2C also resulted in the marked reduction of vessels in the limbus of the cornea when compared to control corneas (Figure 17).

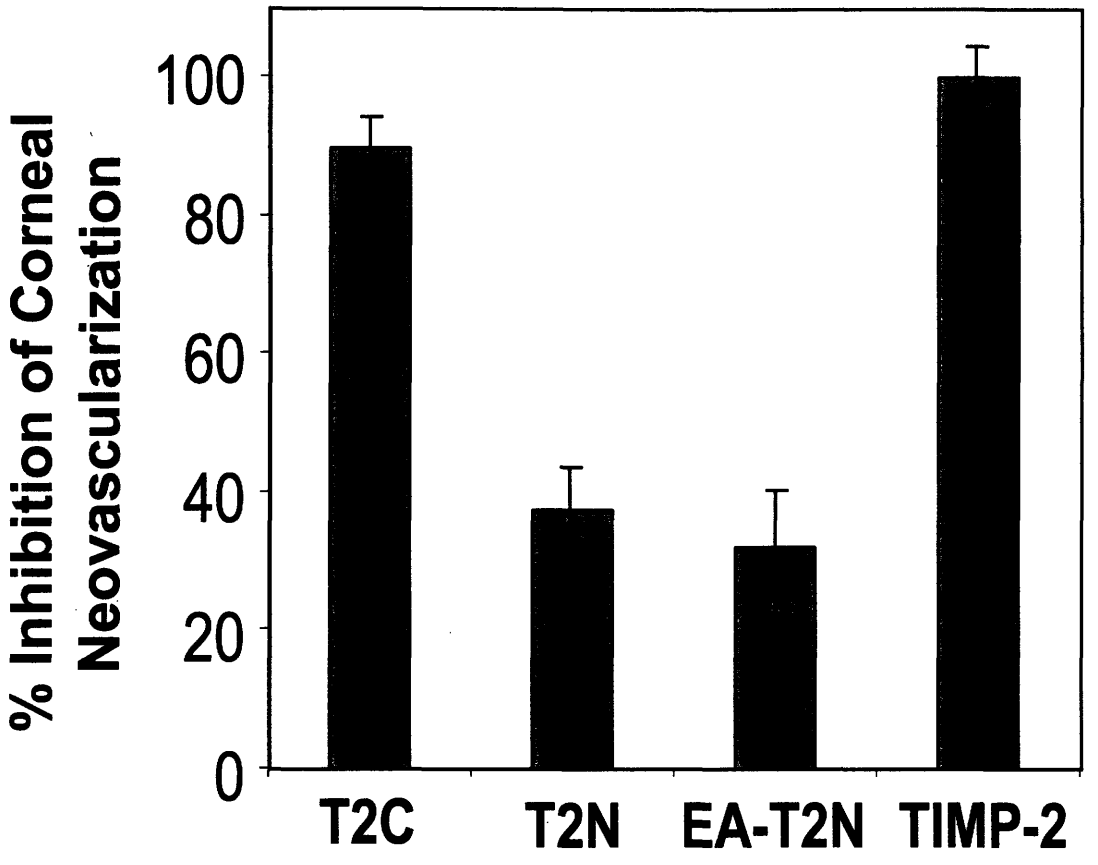


Figure 17. T2C and TIMP-2 are potent inhibitors of angiogenic mitogen-stimulated corneal neovascularization. The area of corneal neovascularization was calculated from the length of the vessels (VL) invading the cornea as well as the clock hours (CH) covered as described by the formula VL x CH x 0.0628. The percent inhibition of corneal neovascularization was calculated from the average inhibition obtained from 18 corneas per treatment group.

Section C: Summary

T2C inhibits normal capillary endothelial cell proliferation, but not MMP activity, while T2N inhibits MMP activity, but not capillary EC proliferation. Both these activities result in the inhibition of angiogenic processes *in vitro*. Interestingly, although both T2N and T2C inhibit embryonic neovascularization in the chick chorioallantoic membrane, only T2C resulted in potent inhibition of corneal neovascularization which is driven by the exogenous addition of an angiogenic mitogen. These results demonstrate that TIMP-2 possesses two distinct anti-angiogenic activities, one that is MMP-dependent and one that is MMP-independent. However, these data also suggest that MMP inhibition alone may not be sufficient to inhibit angiogenesis in conditions where angiogenesis is driven by a marked imbalance of positive and negative regulators, as is the case in pathological angiogenesis.

Chapter 4: Identification of the anti-angiogenic domain of T2C

The previous results demonstrate that the anti-proliferative activity of TIMP-2 is independent of MMP-inhibitory activity, and that T2C, which houses the anti-proliferative activity of TIMP-2, is a potent inhibitor of *in vivo* angiogenesis. The continuing goal of this dissertation was to identify the smallest anti-angiogenic domain.

Section A: Design and synthesis of peptides to various smaller domains of T2C

In order to further isolate the anti-angiogenic activity of TIMP-2 found within T2C, we designed a series of synthetic peptides to various smaller structural domains of T2C and tested these for their ability to inhibit capillary endothelial cell proliferation. The sequence of each of these peptides is based on those secondary structures which are most exposed in the three-dimensional structure of TIMP-2 as determined by Tuttila and coworkers (Tuuttila et al., 1998). The identity of these peptides in relation to the secondary structure is illustrated in Figure 18.

Three peptides were synthesized via Fmoc solid phase synthesis on chlorotrityl resins using DIC/HBTU/HOBT activation to yield peptides as a trifluoroacetic acid salt. Peptides were then further purified using C18 reverse phase HPLC to remove any truncation products. Briefly, 1 mg of dry peptide was resuspended in 1ml of 0.05% trifluoroacetic acid in water and loaded unto the column. Separation was carried out over a two-step gradient with 0.05% trifluoroacetic acid in acetonitrile. The first step increased from 0-20% trifluoroacetic acid/ acetonitrile, followed by a shallow gradient of 20-50% trifluoroacetic acid/ acetonitrile over 60 minutes. Fractions containing the peak of

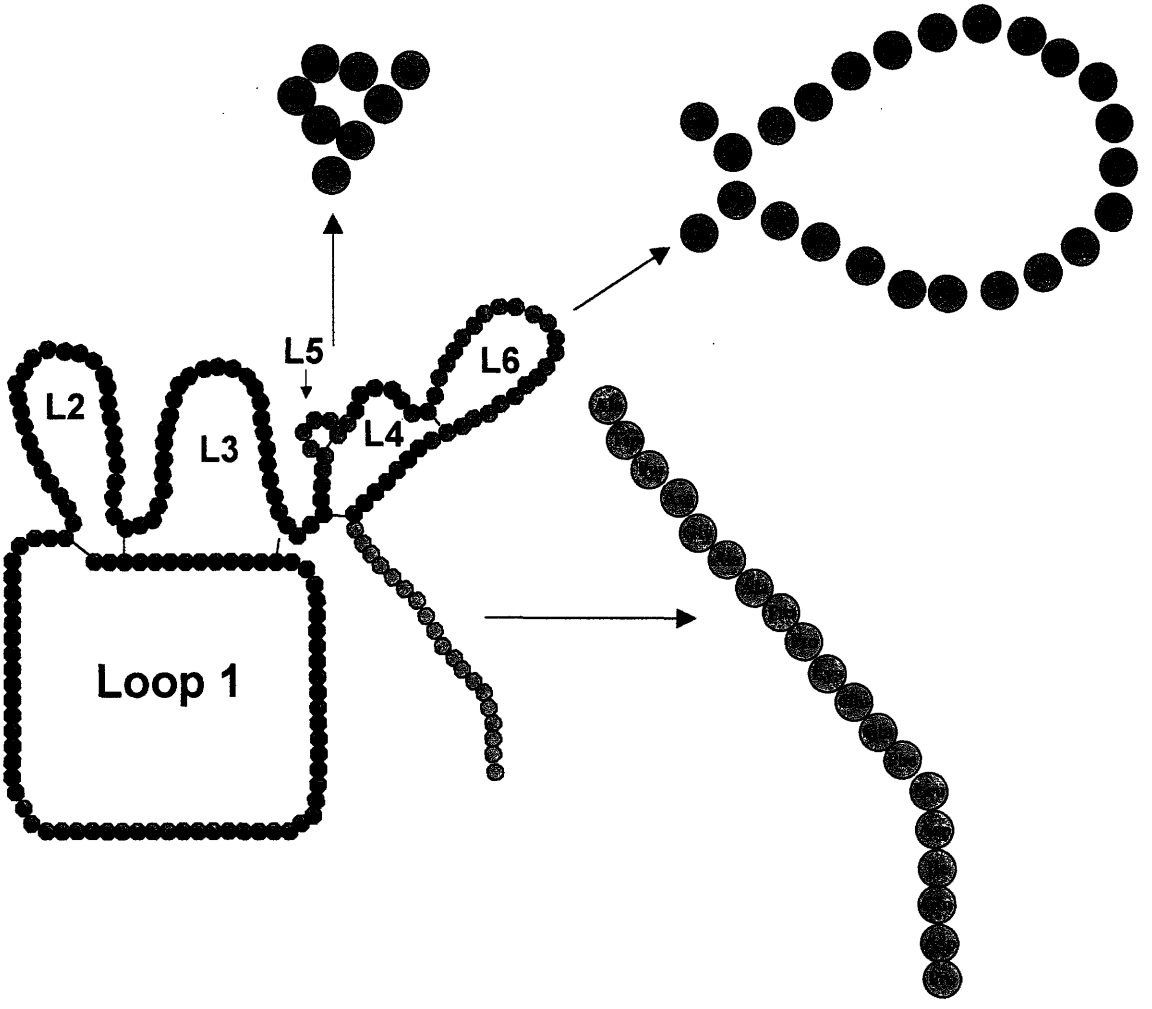


Figure 18. Synthetic peptide design. Peptides representing various smaller structural domains within T2C were synthesized for use in studies aimed at determining the structural elements within T2C responsible for its ability to inhibit angiogenesis.

interest were collected by hand, and identity was verified by mass/charge ration as determined by matrix-assisted laser disorption-time of flight (MALDI-TOF) mass spectroscopy analysis (Figure 19). Once pure, yields were determined using amino acid composition analysis.

Section B: *In vitro* inhibition of EC proliferation by T2C peptides

Purified peptides were tested for their ability to inhibit bFGF-stimulated capillary EC proliferation *in vitro* as described in Chapter 2 of this Thesis. One peptide, corresponding to Loop 6 of intact TIMP-2 with amino acid sequence ECLWMDWVTEKNINGHQAKFFACI, significantly inhibited capillary EC proliferation, while a peptide corresponding to the carboxy-terminal tail (T2-Tail), AWYRGAAPPKQEFLDIEDP, had no effect. A peptide corresponding to Loop 5 of intact TIMP-2, TRCPMIPCYI, stimulated the proliferation of capillary EC (Figure 20). A fourth peptide corresponding to the highly conserved VIRAK sequence of Loop 1 of all four intact TIMPs (amino acid residues 18-22) was used as a random peptide control and resulted in no inhibition of capillary EC proliferation (data not shown). Loop 6 inhibited capillary EC proliferation at doses comparable to T2C, suggesting that Loop 6 is responsible for the anti-proliferative activity of intact TIMP-2.

Reduced and unreduced samples of Loop 6 were analyzed for differences in peptide charge using MALDI-TOF to determine whether the peptide, as tested in all bioassays, contained two free thiols or a disulfide bond. No difference in charge was observed, suggesting that the peptide was in linear conformation. Furthermore, reducing Loop 6 with dithiothreitol (DTT) during the proliferation assays did not result in loss of

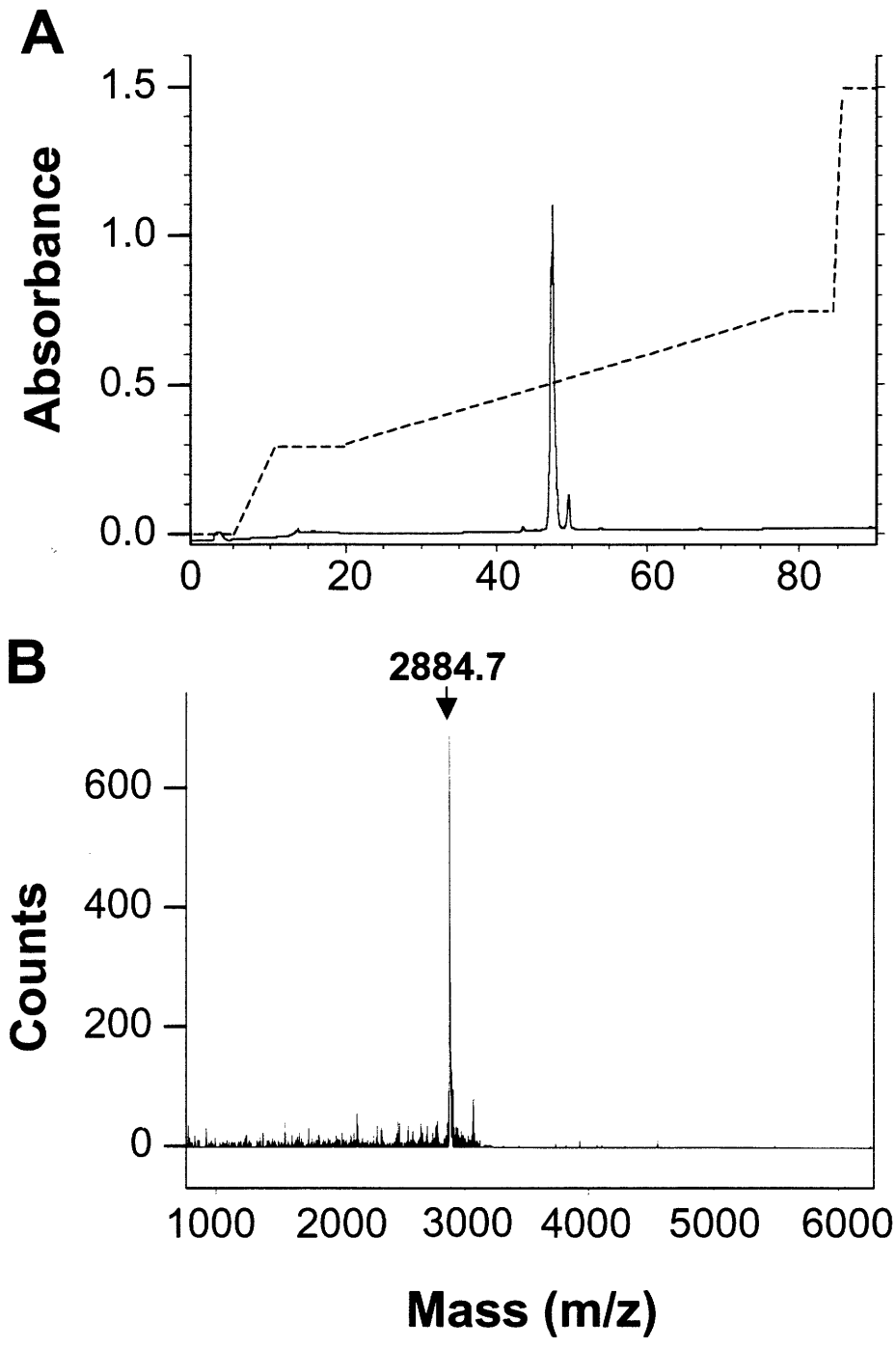


Figure 19. Purification and analysis of synthetic peptides. Synthetic peptides were further purified using C18 reverse phase HPLC and identity was verified by MALDI-TOF mass spectroscopy analysis as described in text. Representative chromatogram of the purification of the Loop 6 peptide by C18 reverse phase HPLC (A) and subsequent analysis by MALDI-TOF (B) are shown.

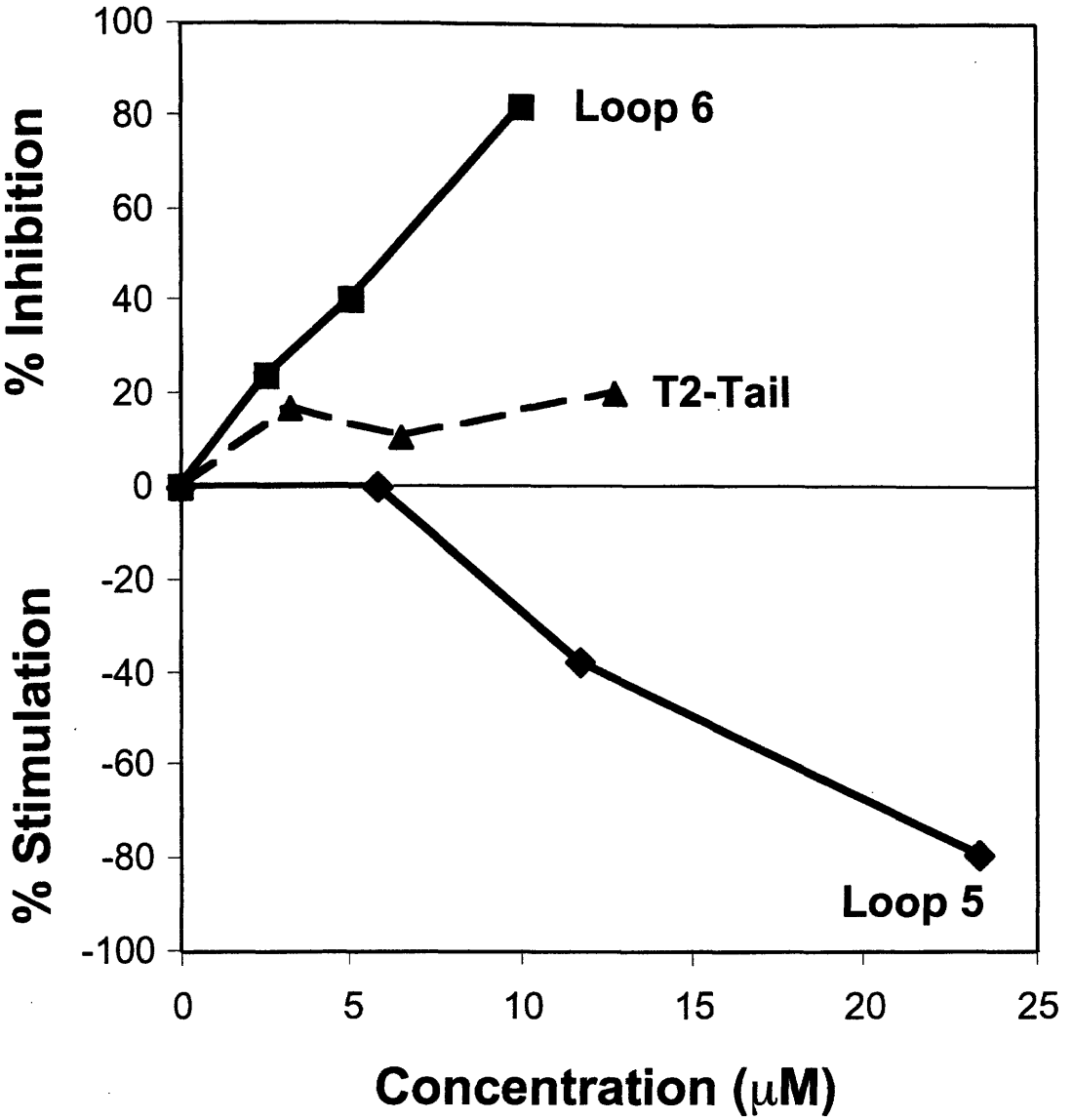


Figure 20. Loop 6 inhibits capillary EC proliferation. To identify the structural determinants of T2C responsible for the inhibition of capillary EC proliferation, synthetic peptides to various sub-domains of T2C were tested in the endothelial cell proliferation assay. Of the peptides tested, only Loop 6 inhibited capillary EC proliferation, while Loop 5 actually stimulated proliferation and the carboxy-terminal tail (T2-Tail) had no significant effect. A representative proliferation assay in which equivalent doses (μg/ml) were tested for each of the peptides is shown above.

inhibitory activity (data not shown). These results suggest that Loop 6 does not require a loop conformation to inhibit angiogenesis.

To confirm that the anti-proliferative effects of Loop 6 are independent of MMP-inhibitory activity, we tested Loop 6 in the radiometric collagen film assay. None of the doses tested, ranging from below the IC_{50} of TIMPs (1.5 nM) to 20-fold higher (150 nM), resulted in any inhibition of MMP activity.

Section C: Loop 6 inhibits both embryonic and mitogen-driven angiogenesis *in vivo*

To determine whether the *in vivo* anti-angiogenic activity of T2C is also retained within Loop 6, Loop 6 was tested in the chick chorioallantoic membrane assay, as described in Chapter 3 of this Thesis. Other synthetic peptides to Loop 5 and to T2-Tail were used as controls. Loop 6 inhibited 77% of all the CAMs tested, and resulted in avascular zones at doses comparable to those tested for T2C and had similar vessel morphology (Figure 21). Neither Loop 5 nor the T2-Tail peptides had any significant effect on neovascularization (data not shown). Interestingly, although Loop 5 stimulated the proliferation of capillary EC *in vitro*, this effect did not translate into the stimulation of neovascularization *in vivo* in this model.

Loop 6 was next tested in the mouse corneal pocket assay to determine whether this peptide alone could also inhibit mitogen-driven angiogenesis *in vivo*. Corneas treated with Loop 6 (3 μg/eye) showed a significant suppression (~80%) of bFGF-stimulated neovascularization when compared to the contralateral control eyes (Figure 22). These results are comparable to those obtained for intact TIMP-2 and T2C (Figure

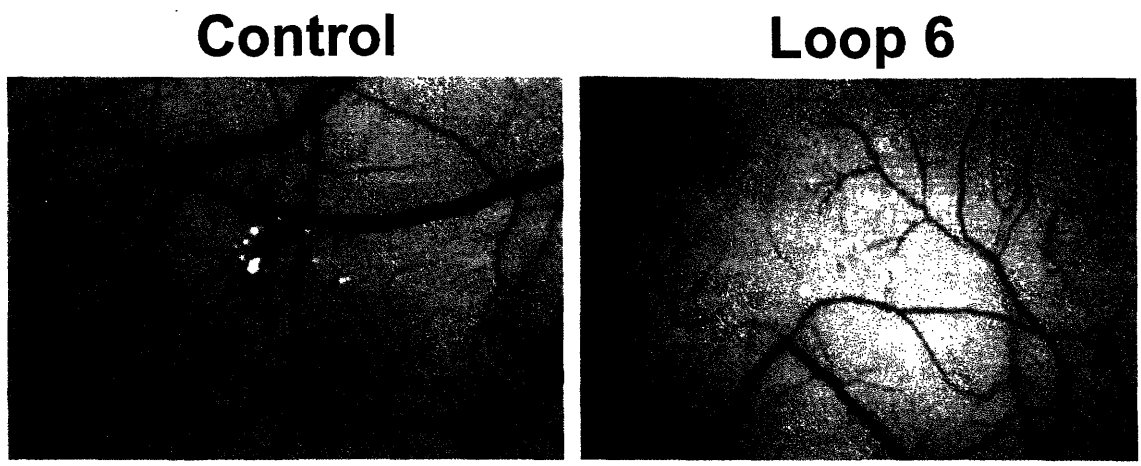


Figure 21. Loop 6 inhibits embryonic angiogenesis in the CAM. To determine whether the anti-angiogenic activity of T2C is housed in Loop 6, Loop 6 was tested for its ability to inhibit embryonic neovascularization in the CAM. Loop 6 resulted in large avascular zones in the CAM as compared to controls. A representative CAM treated with Loop 6 is shown on the right. As with T2C, the remaining vessels in the vicinity of the methylcellulose disc containing Loop 6 were tortuous in appearance.

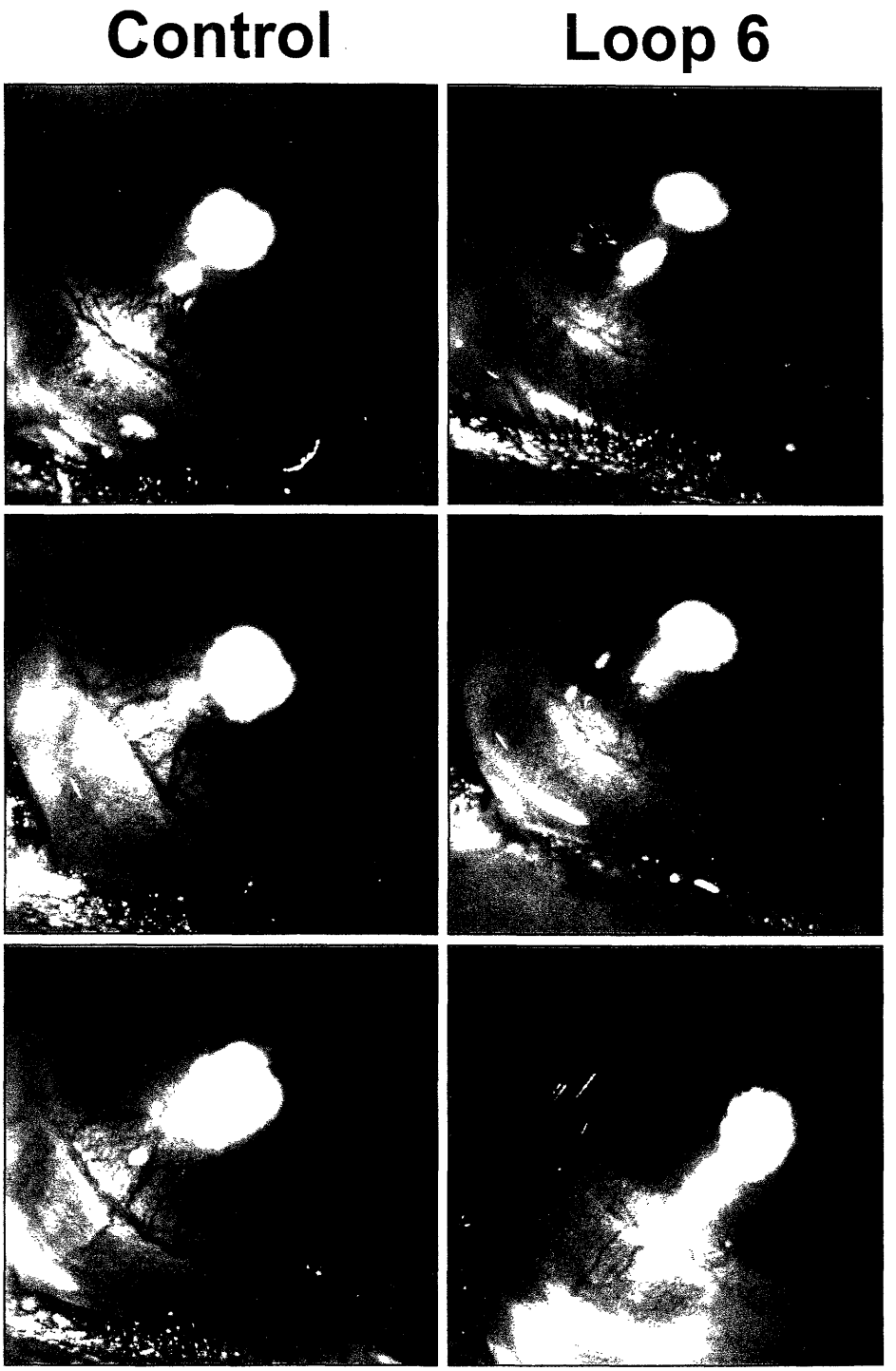


Figure 22. Loop 6 is a potent inhibitor of mitogen-stimulated corneal neovascularization. Representative corneas from three mice treated with Loop 6 are shown on the right hand column. The control corneas for each of the mice shown are on the left column. Loop 6 resulted in 77% inhibition of corneal neovascularization.

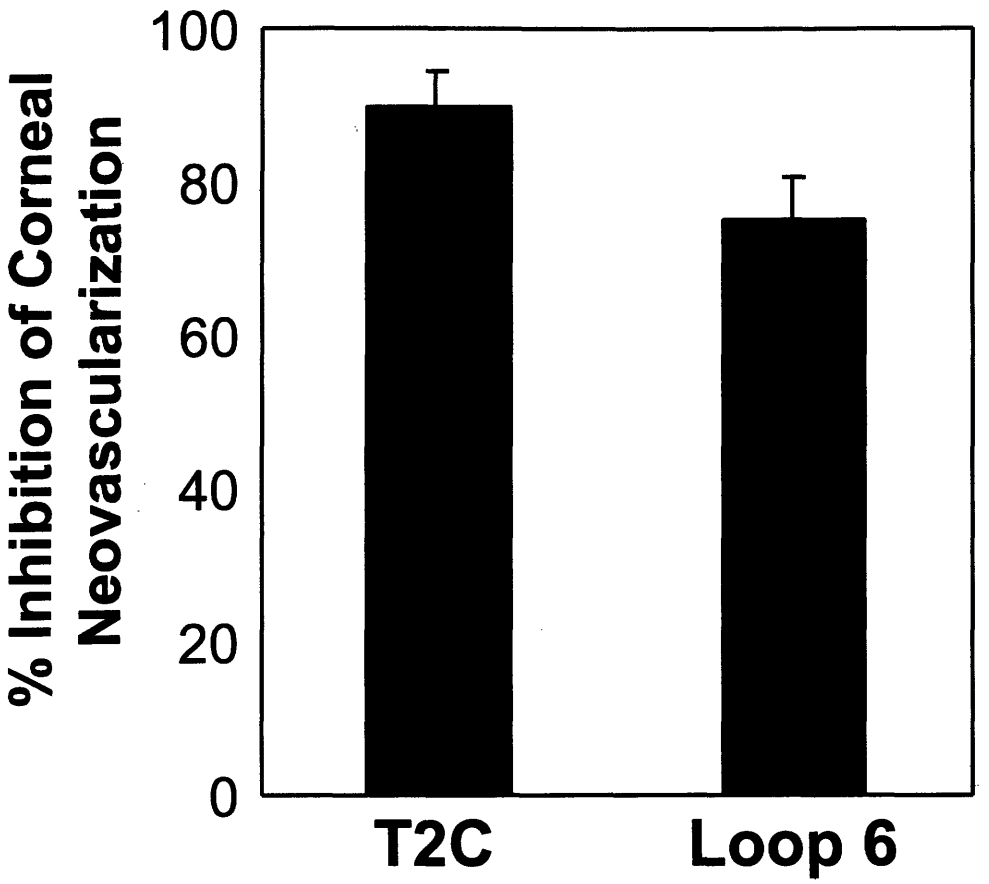


Figure 23. Loop 6 is a potent inhibitor of *in vivo* angiogenesis in the mouse corneal pocket assay. The area of corneal neovascularization and percent inhibition were calculated as previously described in text. The results were obtained from 18 corneas per group. Loop 6 resulted in potent inhibition of mitogen-stimulated corneal neovascularization, which was statistically the same as that observed in corneas treated with T2C.

23). As with T2C, the density of the vessels around the limbus of the corneas was also markedly decreased (Figure 22). These results are significant because they demonstrate that the anti-proliferative activity of Loop 6 within TIMP-2 is independent of MMP inhibitory activity and that it is a powerful inhibitor of bFGF-mediated angiogenesis *in vivo*.

Section D: Summary

The results presented in this chapter demonstrate that the anti-proliferative activity of TIMP-2 can be isolated to a 24 amino acid peptide within T2C. The results not only identify the amino acid residues responsible for this activity, but also demonstrate that this activity can exist independent of the parent molecule. Importantly, Loop 6 resulted in potent inhibition of neovascularization *in vivo* in two separate bioassays. The 2.9 kD Loop 6 therefore, represents a novel, potent, small molecular weight inhibitor of angiogenesis.

Chapter 5: MMP-independent inhibition of angiogenesis by Loop 6; Potential mechanisms of action

Given that the anti-angiogenic effects of TIMP-2, which are housed within T2C, and more specifically Loop 6, are independent of MMP-inhibitory activity, the next goal was to begin to examine some of the possible mechanisms of inhibition. Since T2C and Loop 6 inhibit mitogen-stimulated capillary EC proliferation, it is possible that this effect is due to either an arrest in cell cycle progression or an induction of apoptosis, and that either of these effects are the result of a signaling cascade initiated by a receptor-mediated event. The experiments described below explore these possibilities.

Section A: Loop 6 inhibits cell cycle progression

To determine whether the anti-proliferative effect of Loop 6 on capillary EC is due to the inhibition of cell cycle progression, capillary EC were synchronized by serum starvation and then stimulated with bFGF and treated with an IC_{50} dose of Loop 6 or an equivalent dose of the T2-Tail peptide, which did not inhibit capillary EC proliferation, as a negative control. After 16 hours, cells were trypsinized, fixed in ethanol, stained with propidium iodine and analyzed by flow cytometry for DNA content. Cells treated with Loop 6 showed a marked reduction in the number of cells in G2/M as compared to bFGF controls or cells treated with T2-Tail peptide (9.53%, 39.7% and 34.6%, respectively) as well as an increase in the number of cells in G1 (45.6%, 26.8% and 27.8%, respectively). However, the number of cells in S-phase did not change significantly, suggesting that Loop 6 may suppress S-phase transition. These results are consistent with those

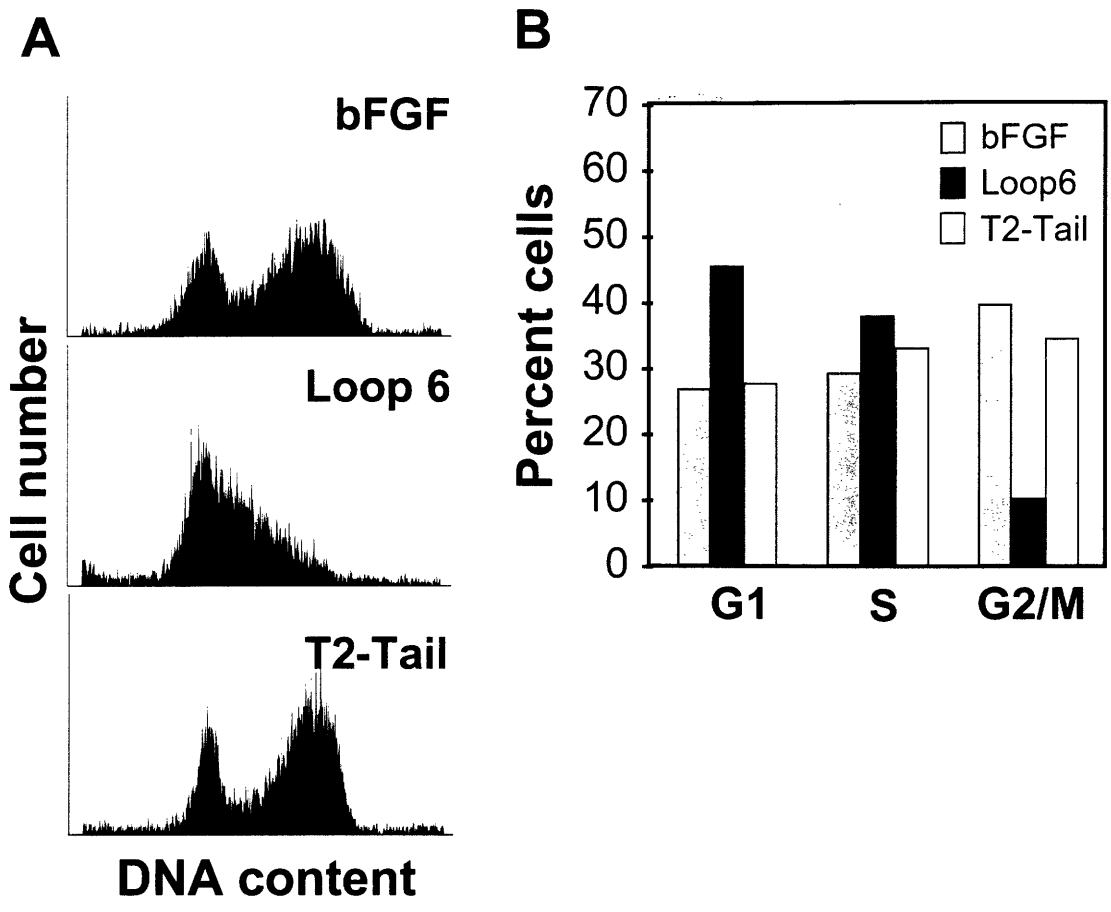


Figure 24. Loop 6 inhibits cell cycle progression. Representative histogram of FACS analyses of the effects of Loop 6 on cell cycle are shown in (A). Loop 6, at doses which result in 50% inhibition of EC proliferation, resulted in marked inhibition of bFGF-stimulated capillary EC cell cycle progression as compared to cells exposed to bFGF alone or to bFGF and equivalent doses of the T2-Tail peptide. The percentage of cells found at the various stages of the cell cycle are shown in (B). Loop 6 resulted in a decrease in the percentage of cells in G2/M, as well as an increase in the number of cells in G1.

previously reported for microvascular endothelial cells engineered to overexpress protein kinase Cδ in which a block of S-phase transition was observed (Ashton et al., 1999). Representative results from our flow cytometry analyses are shown in Figure 24A. The percentage of cells in each of the various stages of the cell cycle are shown in Figure 24B.

We next examined whether the inhibitory effects of Loop 6 on cell cycle progression were the result of altered levels of various positive and negative regulators of cell cycle. Capillary endothelial cells treated for 16 hours with Loop 6 and bFGF or bFGF alone were lysed in 500 µl lysis buffer (1% SDS in 10 mM Tris-Hcl, pH 7.4). The lysate was collected, boiled for 5 minutes, and placed on ice. Samples were passed over a 22-gauge needle ten times to shear the DNA and centrifuged to remove particulates. Protein concentration was determined using the MicroBCA method. Equal amounts of protein were then resolved by SDS-PAGE and analyzed by western blotting using antibodies to various cell cycle associated proteins, including cyclins D1, E and A and the cell cycle inhibitor p27.

No significant differences were observed in the levels of Cyclins D1, E or A (Figure 25). Interestingly, p27, a negative regulator of cell cycle progression, was found to be significantly increased in cells treated with Loop 6 (Figure 25). Previous studies have shown that overexpression of p27 in endothelial cells results in a block in cell cycle progression (Goukassian et al., 2001), and that the cell shape-dependent inhibition of capillary EC proliferation is associated with a failure to down-regulate p27 (Huang et al., 1998). Ashton and coworkers have also shown that the anti-proliferative effect of protein

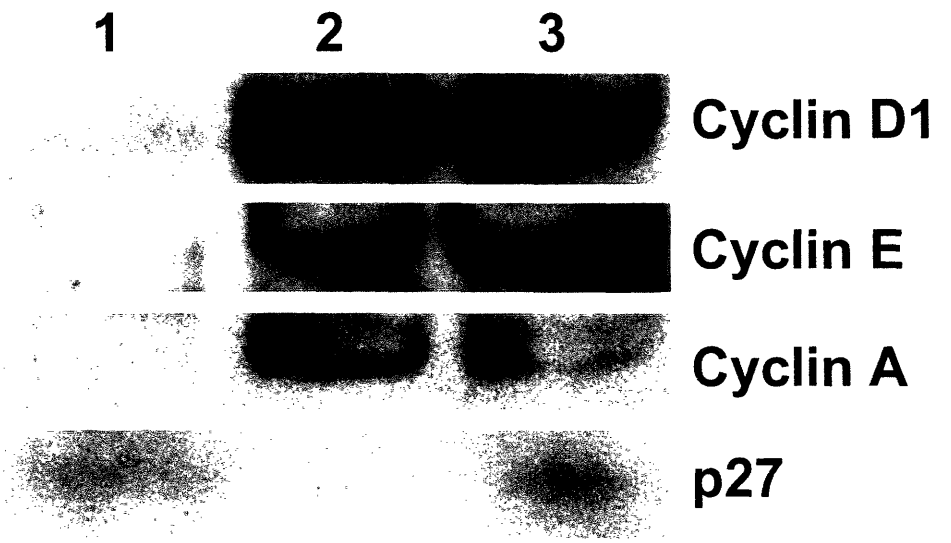


Figure 25. Loop 6 results in increased levels of the cell cycle inhibitor p27. Western analysis of cell cycle associated proteins in the lysates of serum starved capillary EC (Lane 1), bFGF-stimulatedcontrols (Lane 2) and of bFGF-stimulated capillary EC treated with Loop 6 (Lane 3) demonstrate that the inhibition of cell cycle progression by Loop 6 is not the result of decreases in levels of Cyclins D1, E, or A but to, at least in part, an increase in the levels of p27.

kinase Cδ on endothelial cells is the result of increased levels of p27 despite a lack of change in the levels of Cyclins D1, E, and A (Ashton et al., 1999).

Section B: Loop 6 does not induce apoptosis

The next set of experiments examined whether the anti-angiogenic effects of Loop 6 were due to induction of apoptosis in capillary endothelial cells. bFGF-stimulated capillary EC were treated with the IC_{50} dose of Loop 6 for 6 hours and apoptotic nuclei were detected using terminal deoxynucleotidyl transaferase dUTP nick-end labeling (TUNEL) as well as 4,6-diamidino-2-phenylindole (DAPI) staining. No apoptotic nuclei were observed in the bFGF treated control cells nor in Loop 6 treated capillary EC (Figure 26), suggesting that Loop 6 is not an inducer of apoptosis. Wortmannin, which has previously been shown to induce apoptosis of capillary EC (Flusberg et al., 2001), was used as a positive control (Figure 26). All experiments were performed in duplicate, and representative fields of each sample are shown in Figure 28.

Section C: Inhibition of EC proliferation by Loop 6 is not the result of direct competition with bFGF for binding to its receptor.

Given that Loop 6 inhibits bFGF-driven endothelial cell proliferation, we asked whether Loop 6 could directly compete with bFGF. Capillary EC were treated with increasing concentrations of bFGF in the presence of Loop 6 at a dose which results in approximately 50% inhibition (7 μM), and found that the anti-proliferative effect of Loop

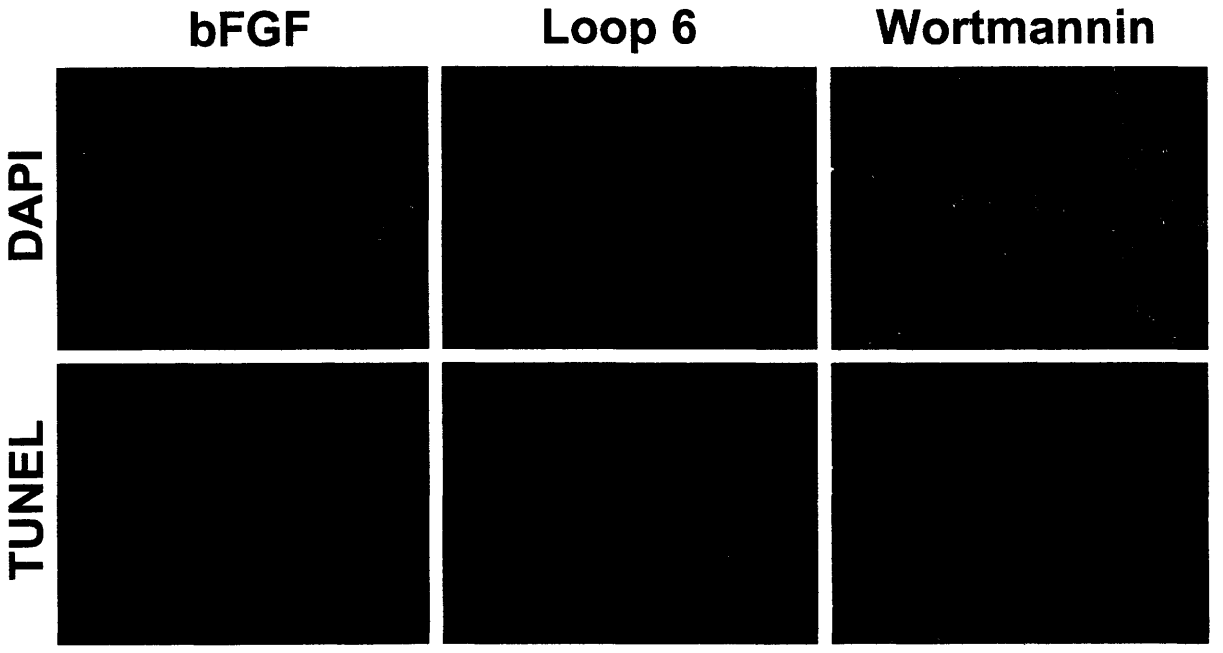


Figure 26. Loop 6 does not induce apoptosis of capillary EC. To determine whether Loop 6 induces apoptosis, capillary EC were treated with Loop 6 and apoptotic nuclei detected by TUNEL staining. Apoptotic nuclei were not observed when capillary EC were stimulated with bFGF or treated with a dose of Loop 6 which results in 50% inhibition of EC proliferation. 100 mM Wortmannin was used as a positive control for TUNEL staining.

6 is not abrogated by increasing doses of bFGF (Figure 27), suggesting that Loop 6 is not a competitor of FGF.

Section D: Loop 6 binds to the capillary EC surface

As described in Chapter 1, TIMP-2 is required for cell surface activation of pro-MMP-2 by MT1-MMP. Given this involvement of TIMP-2 in the activation of pro-MMP-2 and its direct binding to MT1-MMP, the existence of any other cell surface receptors have largely been ignored. However, Hayakawa *et al.*, described the existence of two cell surface receptors on Raji cells, one with high affinity and one with low affinity, although their identity was never elucidated (Hayakawa et al., 1994). Furthermore, in binding studies where excess cold T2N was used as a competitor for cell surface binding of ^{125}I-labeled TIMP-2, T2N was shown to be an order of magnitude weaker than cold TIMP-2 in competing for binding (Ko et al., 1997; Zucker et al., 1998). The failure of T2N to completely compete for TIMP-2 binding suggested that T2C also binds to the cell surface. It has been suggested that T2C might interact with the PEX domain of MT1-MMP prior to binding of pro-MMP-2 although this has never been demonstrated (Ko et al., 1997). This possibility notwithstanding, the binding affinities described by Hayakawa and coworkers on Raji cells suggest that at least one more cell surface receptor of TIMP-2 exists (Hayakawa et al., 1994). That the high affinity receptor is MT1-MMP would be consistent with the affinity observed in other cell types where one receptor is observed. It is possible that the low affinity binding represents a second, as of yet unidentified, receptor of TIMP-2.

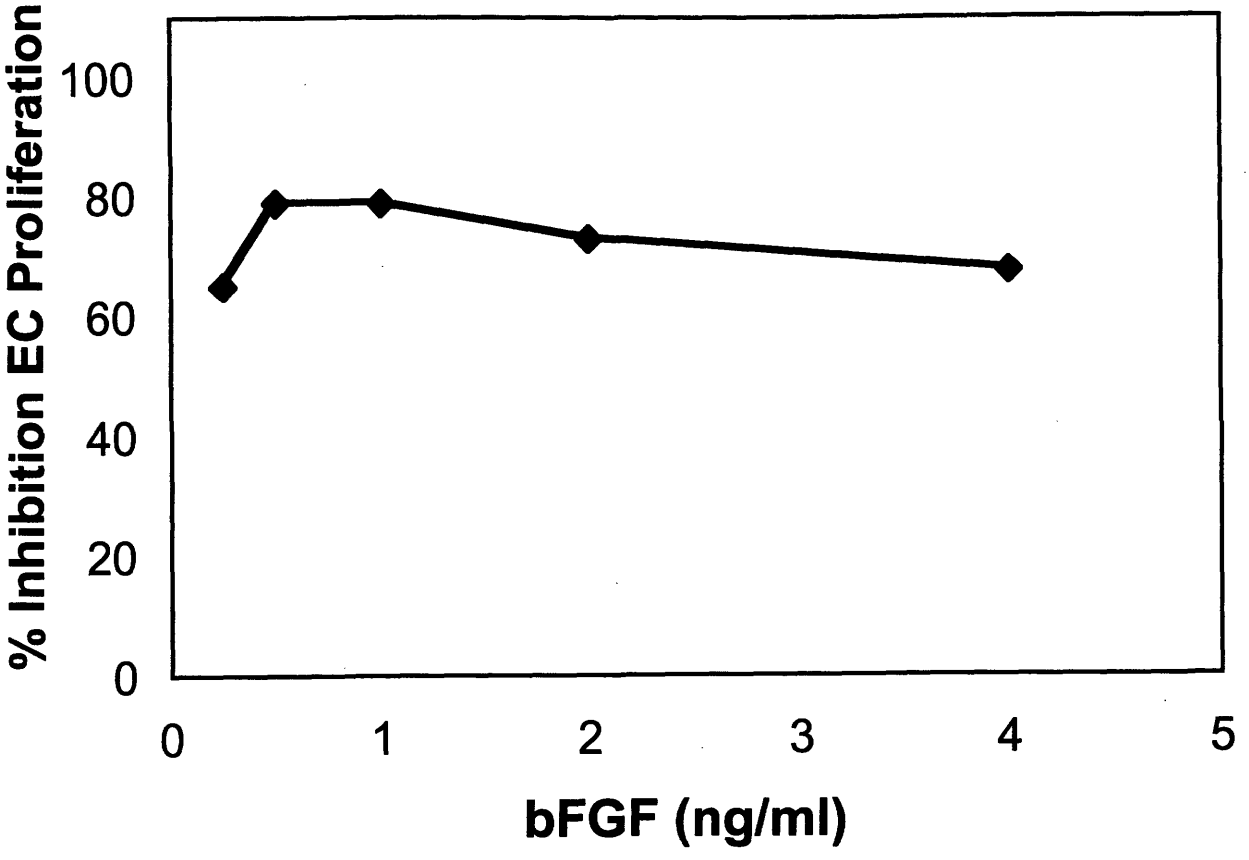


Figure 27. Loop 6 does not compete with bFGF for binding to its receptor. To determine whether Loop 6 inhibits capillary EC proliferation by competing with bFGF for binding to its receptor, capillary EC were treated with a steady dose of Loop 6 while bFGF concentrations were increased to saturation. Increased bFGF concentrations did not result in loss of inhibitory activity of Loop 6, suggesting that Loop 6 does not compete with bFGF for binding to its receptor.

In order to determine whether the anti-proliferative effect of TIMP-2, found within Loop 6, is the result of a receptor-mediated event, we assessed the ability of Loop 6 to bind to the capillary endothelial cell surface. Although we succeeded in labeling Loop 6 with ^{125}I, the reaction was inefficient given the lack of tyrosine residues. Iodination was instead accomplished by the addition of ^{125}I to the histidine residue of Loop 6, leaving one lysine residue available for crosslinking to any interacting cell surface protein. Since chemical crosslinking reaction are inefficient in that only 5% of all interacting proteins are actually crosslinked, complex formation of ^{125}I -Loop 6 with cell surface proteins was difficult to detect. However, our studies suggested that at least three complexes were formed (data not shown).

Given the technical issues associated with the iodination and crosslinking of Loop 6, we next examined whether T2C could bind to the EC cell surface. Briefly, T2C was labeled with ^{125}I as described in 1996 by Soker *et al.* (Soker et al., 1996), using IODO BEADS and the iodinated protein was then purified from free label using a G-25 Sepharose size exclusion column. ^{125}I-T2C having a specific activity of 70,000 CPM/ng was diluted in 4 ml of binding buffer (50 ml DMEM, 0.1% gelatin, 20 mM HEPES), added to capillary endothelial cells in the presence of bFGF, and allowed to incubate for two hours. Interacting proteins were crosslinked with Bis(Sulfosuccinimidyl) suberate (BS^3), the cells lysed and the cellular membrane fraction separated by SDS-PAGE.

Autoradiography of the gels was used to determine the presence and molecular weight of putative receptor/TIMP-2 complexes. Figure 28 shows the results of the EC

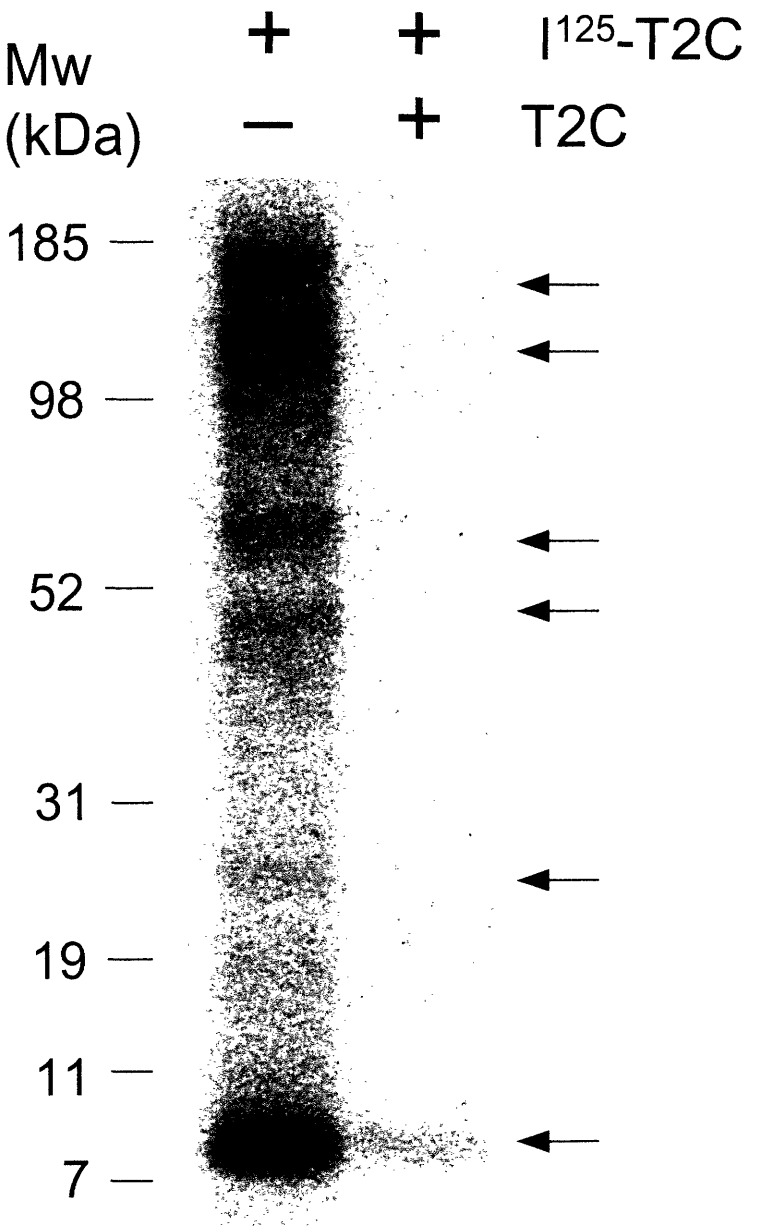


Figure 28. T2C binds to the cell surface of capillary EC. ^{125}I-labeled T2C bound the EC cell surface. Five potential T2C binding proteins are observed (Lane 1) which are competed away by 100 molar excess unlabeled T2C (Lane 2). Experiments with ^{125}I- labeled Loop 6 yielded similar results.

surface binding experiments of ^{125}I-T2C and demonstrates the existence of various candidate cell surface receptors. A 100-fold molar excess of cold T2C successfully competed for cell surface binding. Although experiments using ^{125}I-Loop 6 yielded similar results, the labeling of Loop-6 is restricted to a single histidine residue per molecule and the resulting specific activity was extremely low, making the visualization of cross-linked products more challenging than with ^{125}I-T2C. The identity of putative receptors shown in Figure 28 remains to be determined.

Section E: Summary

The results presented in this Chapter demonstrate that Loop 6 inhibits capillary endothelial cell proliferation, at least in part, by inhibiting cell cycle progression and not by inducing apoptosis. The inhibtion of cell cycle progression was associated with an increase in p27 levels, although the cyclins were not affected. Loop 6 does not compete with bFGF for binding to its receptor. Importantly, cell surface interactions were observed for cells treated with ^{125}I-labeled Loop 6 and T2C. In cross-linking studies of ^{125}I-labeled T2C binding to the capillary endothelial cells, analysis of the cross-linked products by SDS-PAGE and autoradiography revealed the existence of at least five distinct complexes. These associations are specific in that 100-fold excess cold T2C completely competed away binding. Although we obtained similar results with Loop 6, iodination and subsequent cross-linking of this peptide proved difficult given that ^{125}I-labeling was only possible at its single His residue and that crosslinking, which is often inefficient, is only possible at its one Lys residue.

Chapter 6: TIMP-4 inhibits capillary endothelial cell migration

Since the newest member of the TIMP family, TIMP-4, was first cloned in 1996 (Greene et al., 1996) very little work has been done to characterize its possible role in modulating angiogenesis. What is known is that TIMP-4 is capable on inhibiting MMP activity with similar kinetics as the other TIMPs, and that it shares the ability to inhibit the invasiveness of cancer cell lines (Greene et al., 1996; Wang et al., 1997). These findings are in accordance to similar studies using other members of the TIMP family. Interestingly, TIMP-4 is most similar to TIMP-2, raising the possibility that it too could inhibit angiogenesis by inhibiting EC proliferation. As discussed in Chapter 1, very little is known about the effect of TIMP-4 in angiogenesis. Given the continued interest in the role of MMPs and their inhibitors in the process of angiogenesis, a goal of this research was characterize the anti-angiogenic activities of TIMP-4, as described in Table IV.

Section A: Cloning, expression and purification of TIMP-4

Human *TIMP-4* was cloned from a human heart cDNA library using high-fidelity PCR and TIMP-4 specific primers (Figure 29A). The PCR product was cloned into the PICZαA *Pichia pastoris* expression vector as described for TIMP-2 earlier in this dissertation. Its sequence was verified and the construct linearized for insertion into the *Pichia* genome. Positive clones carrying the *TIMP-4* gene were selected as previously described and expression conditions optimized. A single clone expressing TIMP-4 was chosen for subsequent studies and its identity was verified by N-terminal sequencing.

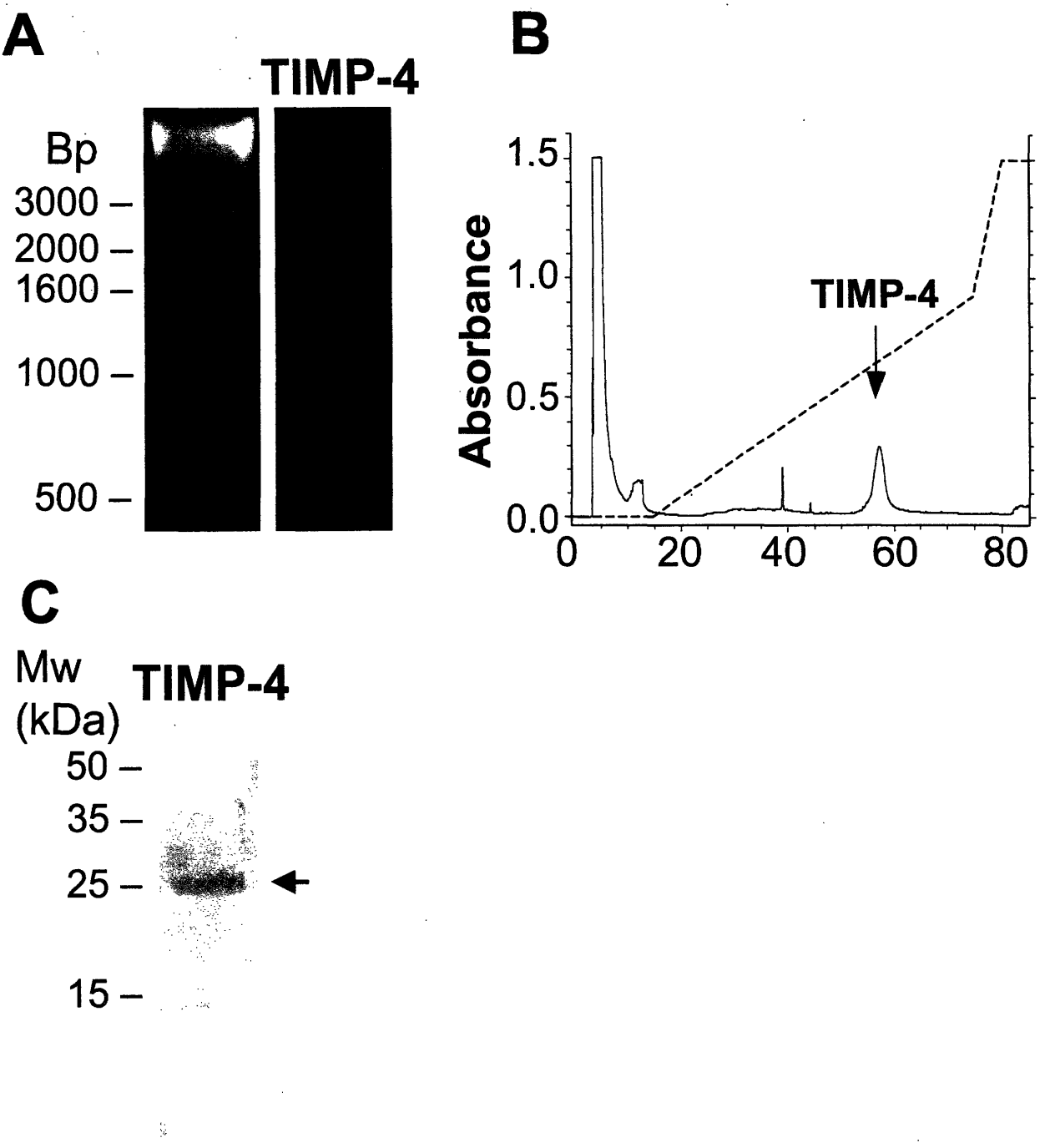


Figure 29. Cloning, purification and expression of TIMP-4. Human *TIMP-4* was cloned from a heart cDNA library using specific primers to the mature form of TIMP-4. The PCR product (A) was cloned into the pPICZαA expression vector as described and inserted into the yeast genome. Expressed TIMP-4 was purified using His-affinity chromatography followed by C4 reverse phase HPLC. A representative HPLC chromatogram is shown in (B). Purification was monitored by silver-stained SDS-PAGE as described. An example of a silver-stained SDS-PAGE gel of TIMP-4 is shown in (C).

Interestingly, TIMP-4 expression was considerably lower (80 μg/L) than that of TIMP-2 or the TIMP-2 domains and protein stability was an issue. In fact, once harvested, protein degradation occurred so rapidly that protein could no longer be detected by SDS-PAGE 24 hours later even when protein was kept at 0° C. Additionally, expressed TIMP-4 did not survive freeze/thaw cycles. Ultimately, our purification protocol had to be designed around this issue, which meant that any TIMP-4 protein expressed had to be fully purified the same day it was harvested.

After initial purification by His affinity chromatography, the first approach attempted to fully purify TIMP-4 used size exclusion chromatography as a second purification step. One of the benefits of this approach was that the protein would be eluted in a buffer compatible with all the bioassays we needed to perform, in this case PBS. However, the large volume in which the protein eluted made a second concentration step necessary, and given that we knew that the protein would quickly degrade if not dry, our best option was to dialyze against water to remove excess salt and then lyophilize the protein. Unfortunately, dialysis against water also seemed to result in complete loss of the protein as judged by the inability to detect a protein band by SDS-PAGE.

Ultimately, a modified strategy of the one employed for TIMP-2 and the TIMP-2 domains, which included C4 reverse phase HPLC as a second purification step, was used, but deleted the dialysis step after chromatography. Although reverse phase chromatography is performed at very low pH values and is therefore not an optimal condition for protein purification, it turned out to be the most feasible second purification step in that it allowed for processing of the most protein in the least amount of time.

Because both acetonitrile and TFA are volatile, the dialysis step was not required, and the protein could be dried using a speed vac without the complication of excess salt. A sample chromatogram from the purification of TIMP-4 by C4 reverse phase HPLC is shown in Figure 29B. Sample purity was assessed by silver-stained SDS-PAGE as previously described (Figure 29C). Appropriate acetonitrile/TFA controls treated in the same way as TIMP-4 were included in all bioassays to ensure that removal of the dialysis step did not result in buffer interference in any of the assays.

Section B: Inhibition of MMP activity by TIMP-4

TIMP-4 activity was confirmed using the radiometric collagen film assay, as previously described, to test for inhibition of MMP activity. As expected TIMP-4 inhibited MMP activity at nanomolar concentrations, with an IC_{50} of approximately 15 nM. Representative results are shown in Figure 30. These results demonstrate that we have succeeded in expressing and purifying active TIMP-4, and are in accordance to published results where the IC_{50} varies anywhere from 3 nM to 83 nM depending on the MMP tested and the system used (Liu et al., 1997).

Section C: TIMP-4 inhibits migration but not proliferation of capillary EC

Given the high degree of homology between TIMP-2 and TIMP-4, we hypothesized that TIMP-4 might share capillary EC anti-proliferative activity with TIMP-2. TIMP-4 was tested for its ability to inhibit EC proliferation as described above. The results demonstrate that, contrary to our hypothesis, TIMP-4 did not significantly inhibit EC

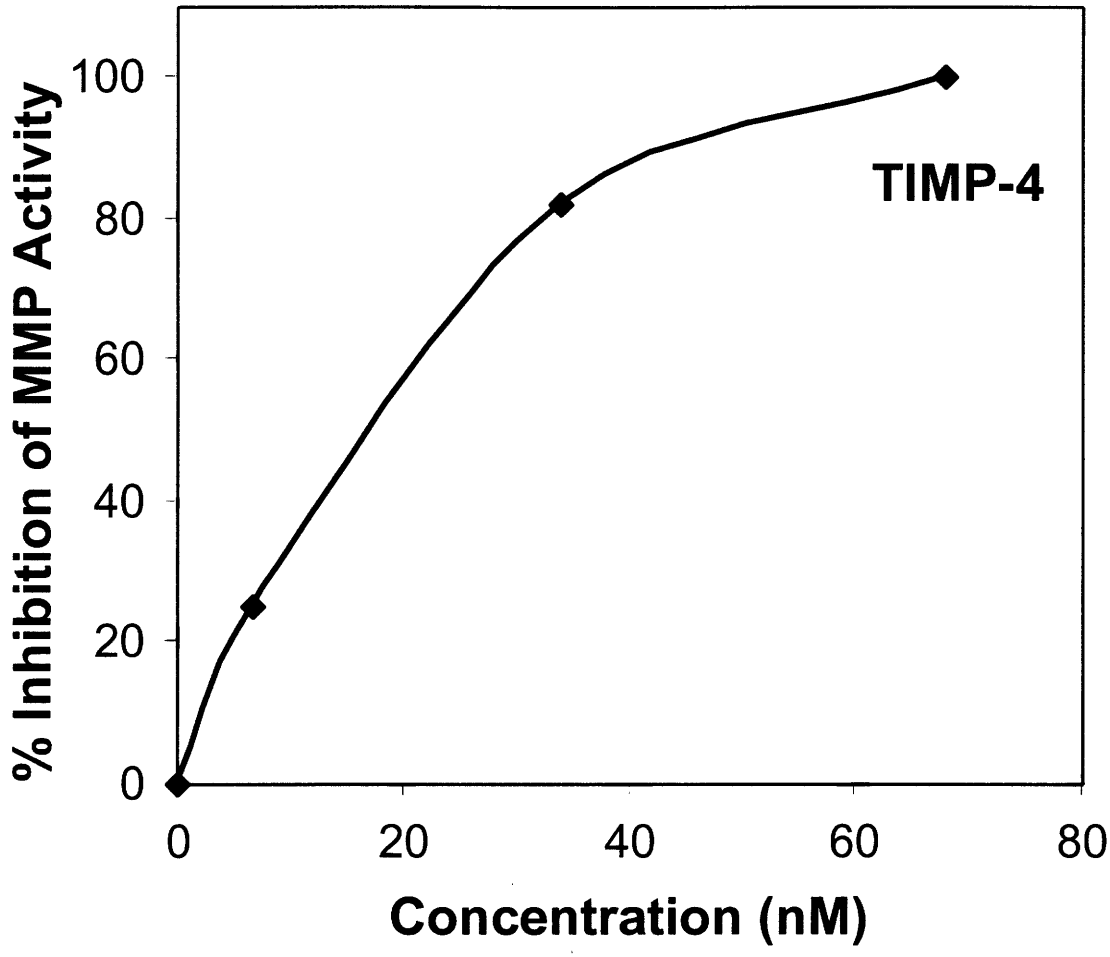


Figure 30. TIMP-4 inhibits MMP activity. To verify that the expressed TIMP-4 was correctly folded and retained MMP inhibitory activity, TIMP-4 was tested in the radioactive collagen film assay, as described. TIMP-4 inhibited MMP activity with an IC_{50} of approximately 15 nM.

proliferation. A representative assay is shown in Figure 31. Interestingly, TIMP-4 has been shown to inhibit Wilm's tumor cell growth *in vitro* at doses as low as 1 nM (Celiker et al., 2001), but not the proliferation of breast tumor cells at any dose tested (Wang et al., 1997). These results suggest that the growth modulating effects of TIMP-4 might be cell specific.

Since, as described in Chapter 5, Loop 6 of TIMP-2 was identified as the anti-proliferative site of that molecule, a comparison of the sequences of TIMP-2 and TIMP-4 was undertaken. Although TIMP-2 and TIMP-4 share approximately 50% overall homology, Loop 6 of TIMP-4 (T4L6) shares less than 25% homology with Loop 6 of TIMP-2. A synthetic peptide corresponding to Loop 6 of TIMP-4 was synthesized and purified and tested for its ability to inhibit capillary EC proliferation. Although a modest anti-proliferative effect was observed, the dose required to elicit this effect was very high (33 μM), and was approximately 5 times higher than the IC_{50} of Loop 6 of TIMP-2. Representative results are shown in Figure 32. It is possible that the residues that TIMP-2 and TIMP-4 have in common at Loop 6 are responsible for some of the anti-proliferative effects, but that the difference in amino acid residues results in a reduction in the specific activity of the T4L6 peptide.

Given that all TIMPs tested to date can inhibit the migration of capillary EC, we next tested TIMP-4 in an *in vitro* migration assay. To do this, a two-chamber well system was employed in which the upper and lower chambers are separated by a membrane with 8 μm pores through which the endothelial cells can migrate. The membranes were coated with fibronectin and after 1 hour 50,000 cells were plated on each of the upper chambers.

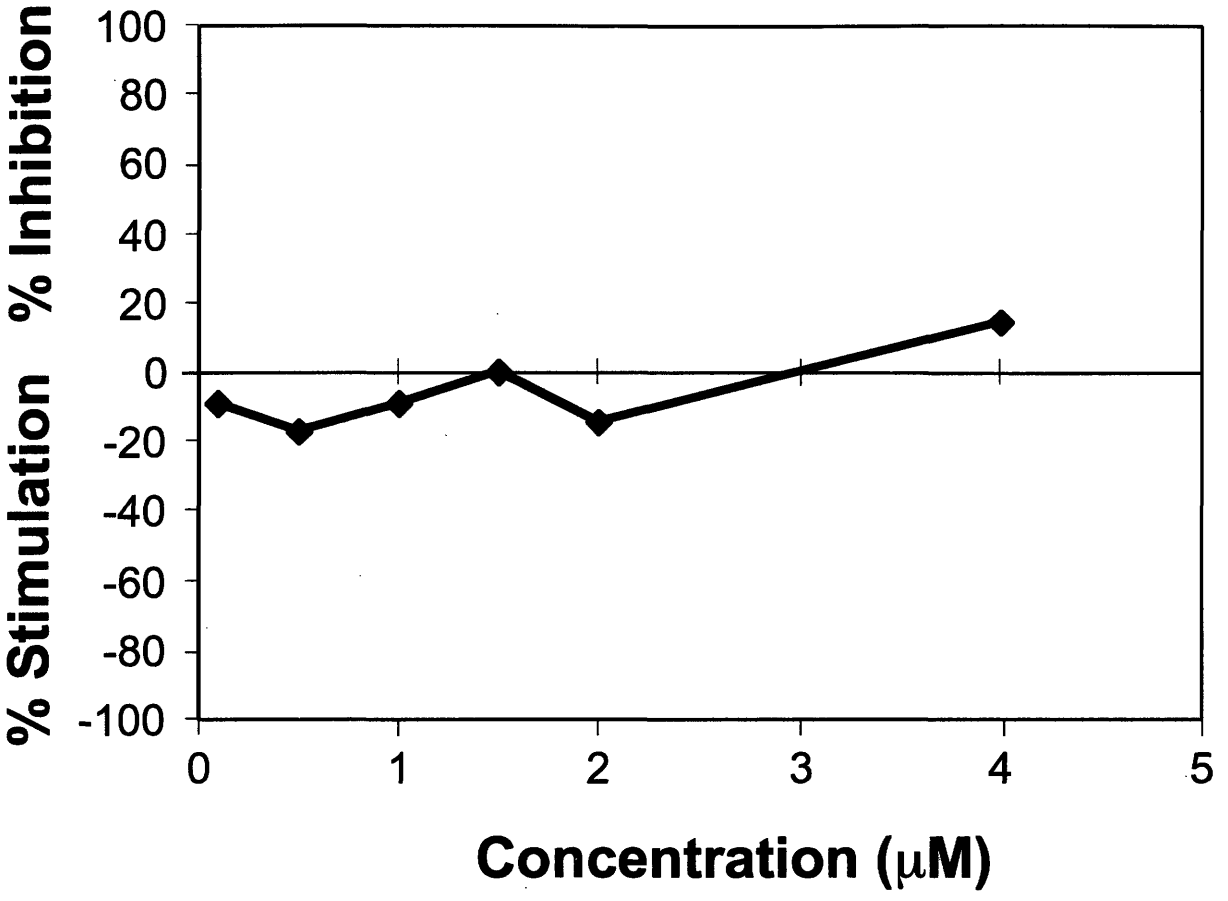


Figure 31. TIMP-4 does not inhibit capillary EC proliferation. To determine whether TIMP-4 shares anti-proliferative activity with TIMP-2, TIMP-4 was tested for its ability to inhibit capillary EC proliferation, as described in text. TIMP-4 had no significant anti-proliferative activity.

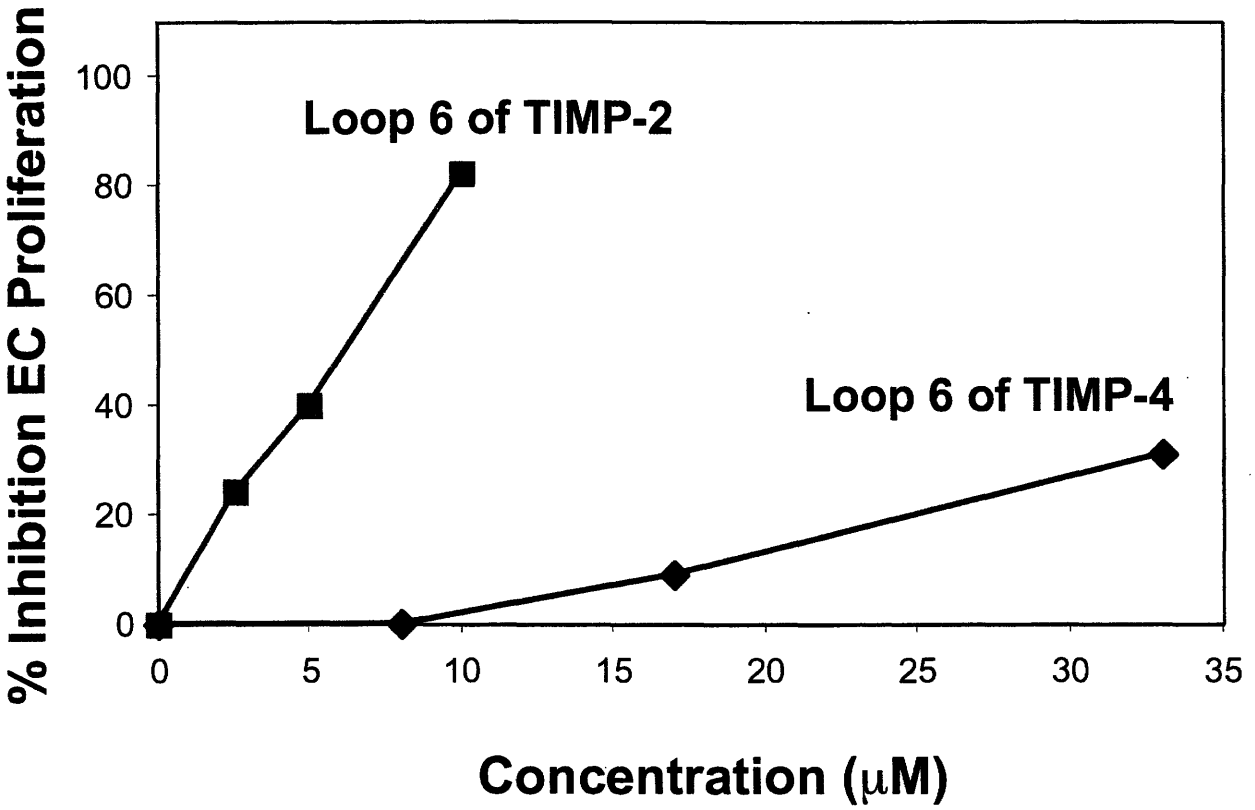


Figure 32. Loop 6 of TIMP-4 does not inhibit capillary EC proliferation. Since the anti-proliferative activity of TIMP-2 was found to be housed, at least in part, in Loop 6, Loop 6 of TIMP-4 (T4L6) was synthesized and tested for its ability to inhibit capillary EC proliferation. Although a modest inhibitory activity could be detected,the dose of T4L6 requiredwas extremelyhigh and approximately 5-fold higher than those required to achieve 50% inhibition when cells were treated with Loop 6 of TIMP-2.

The cells were allowed to attach to the membrane over 30 minutes and samples were added at various concentrations to the designated wells. Media containing serum plus bFGF was added to the lower chambers to stimulate migration. After 4 hours, the media was aspirated and the cells fixed and stained using the DiffQuik stain kit. Total number of migrated cells was determined from a digital image of each membrane. Dark-stained nuclei were individually marked using the density gradient function of NIH Image, and the total number of cells was determined electronically by the number of particles marked (Figure 33A). Each sample was tested in duplicate. TIMP-4 inhibited capillary EC migration at doses comparable to those reported for TIMP-4 in other cell lines (Liu et al., 1997), with an IC_{50} of approximately 55 nM (Figure 33B).

Section D: TIMP-4 does not inhibit embryonic angiogenesis in the CAM

Interestingly, TIMP-4 has previously been shown to inhibit capillary tube formation *in vitro*, although the dose required for this effect was very high (500 nM) (Lafleur et al., 2002). The authors suggest that the high dose required to inhibit tubulogenesis might be due to the presence of MMPs in the growth medium. These *in vitro* studies underline the importance of assessing the ability of TIMP-4 to inhibit angiogenesis *in vivo*.

Unlike TIMP-2, the results presented here demonstrate that TIMP-4 did not inhibit capillary EC proliferation, its effect on EC migration might be sufficient to inhibit angiogenesis *in vivo*. Therefore, TIMP-4 was tested for its ability to inhibit unstimulated angiogenesis in the CAM. TIMP-4 did not result in any significant inhibition of embryonic angiogenesis as determined by the lack of avascular zones around the

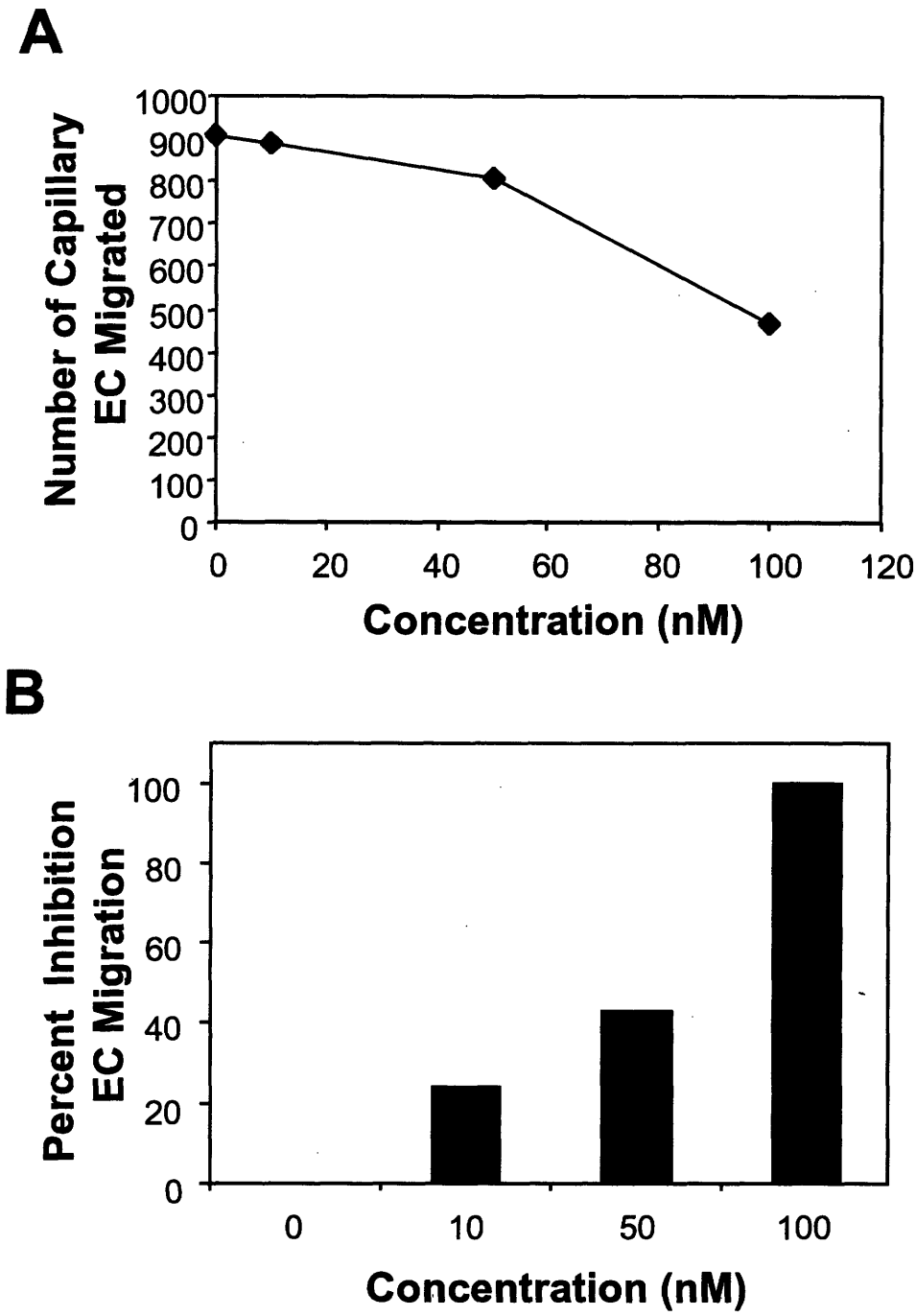


Figure 33. TIMP-4 inhibits capillary EC migration. Given that all the TIMPs tested to date (TIMPs-1, -2, -3) have been shown to inhibit endothelial cell migration *in vitro*, TIMP-4 was tested for its ability to inhibit capillary EC migration. (A) The total number of cells migrating in response to bFGF stimulation was determined as described in text. (B) Percent inhibition was calculated from the total number of cells migrating in response to bFGF stimulation (963 cells) as compared to un-stimulated controls (502 cells), with an IC_{50} of approximately 50 nM and maximal inhibition achieved at 100 nM (463 cells). Doses higher than 100 nM did not result in any significant decrease in cells migrating (data not shown).

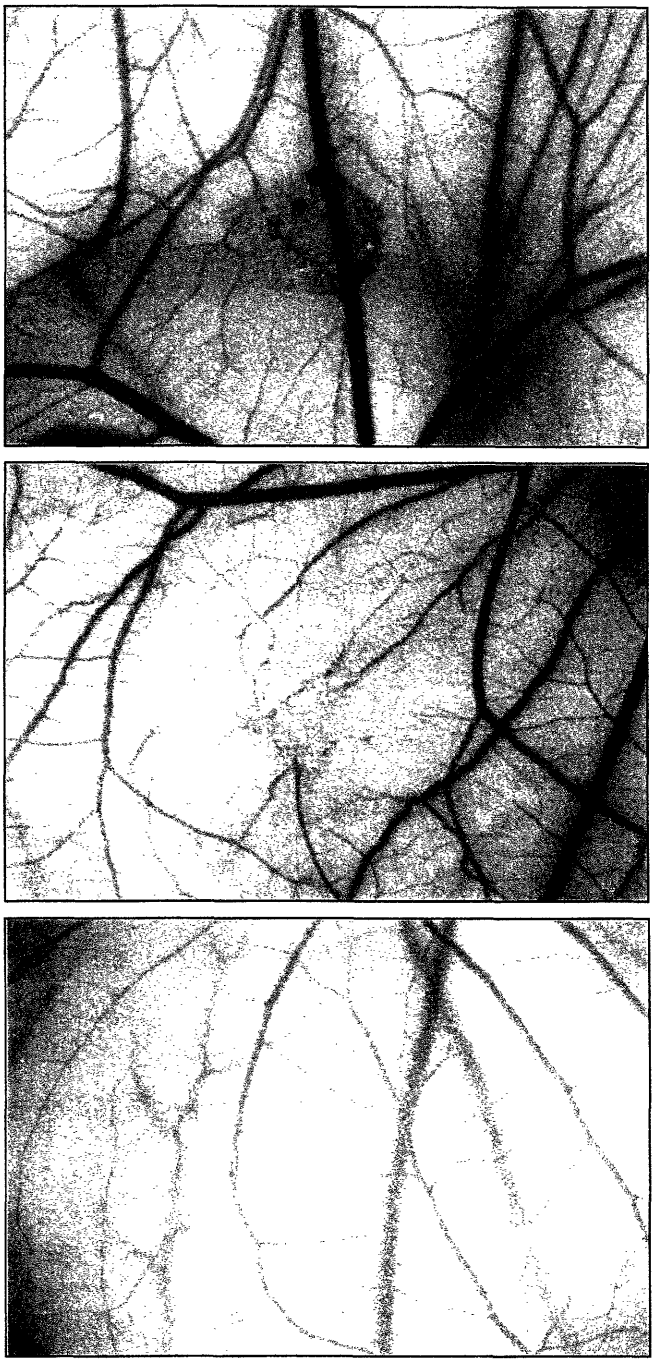


Figure 34. TIMP-4 does not significantly inhibit embryonic angiogenesis in the CAM. TIMP-4 was tested for its ability to inhibit angiogenesis *in vivo* in the CAM assay. TIMP-4 did not result in any significant inhibition of embryonic neovascularization. Three representative CAMs are shown.

methylcellulose discs. Representative CAMs are shown in Figure 34. The inhibition of CAM neovascularization was characterized by a reduction in capillary vessel branching, rather than to the formation of a marked zone of vessel clearance. These results are most comparable to those obtained in CAMs treated with T2N and are consistent with the inhibition of MMP activity, as previously described.

Section E: Summary

The experiments described above demonstrate that TIMP-4, like TIMPs -1, -2, and -3 is an inhibitor of capillary EC migration. However, data presented here demonstrates that TIMP-4 is not an inhibitor of capillary EC proliferation. These results are most like those obtained for TIMP-3 (Anand-Apte et al., 1997). TIMP-4 did not significantly inhibit neovascularization in the CAM and did not result in pronounced zones of vessel clearance, as is observed in CAMs treated with TIMP-2.

Chapter 7: Discussion

Section A: TIMP-2 possesses two distinct anti-angiogenic activities which can be dissociated from each other

It is widely appreciated that TIMPs are multi-functional proteins with respect to cell growth, apoptosis, angiogenesis and other bioactivities (Brew et al., 2000; Baker et al., 2002). Although some of these differences in TIMP activities can be attributed to differences in their affinity for various MMPs and/or their role in MMP activation, other functions appear to be entirely MMP-independent. For example, both TIMP-1 and TIMP-2 have been shown to possess erythroid-potentiating activity (Gasson et al., 1985; Stetler-Stevenson et al., 1992). TIMP-3 has been reported to inhibit tumor necrosis factor-alpha converting enzyme (Amour et al., 1998) as well as inducing apoptosis (Smith et al., 1997; Baker et al., 1999; Bond et al., 2000), while TIMP-1 actually inhibits apoptosis (Guedez et al., 1998b; Guedez et al., 1998a; Li et al., 1999). TIMP-2 has been shown to induce apoptosis in some systems (Lim et al., 1999; Brand et al., 2000) but to have no effect in others (Bond et al., 2000). Of particular interest is the fact that only TIMP-2 has been shown to inhibit capillary EC proliferation (Murphy et al., 1993; Anand-Apte et al., 1997).

Given that all TIMPs can inhibit MMP activity, the ability of TIMP-2 to inhibit capillary EC proliferation has been suggested to constitute a second function of TIMP-2, independent of its MMP-inhibitory activity (Murphy et al., 1993; Hoegy et al., 2001). Based on these findings, we hypothesized that there must be a structural entity unique to

TIMP-2 that might be responsible for the inhibition of mitogen-driven capillary EC proliferation, thereby representing a second anti-angiogenic site within the molecule. In fact, a mutant form of TIMP-2 that lacks MMP-inhibitory activity has recently been shown to inhibit the proliferation of various tumor cell lines (Hoegy et al., 2001). However, no direct evidence of independent structural elements responsible for growth inhibition has been demonstrated prior to this current study (Murphy et al., 1993; Hayakawa et al., 1994; Hoegy et al., 2001). In our structure-function study, we isolate and characterize the MMP-dependent and MMP-independent anti-angiogenic effects of TIMP-2 and demonstrate, for the first time, that these activities are structurally independent. In doing so, we have identified a novel inhibitor of angiogenesis, Loop 6.

The first series of experiments demonstrate that TIMP-2 possesses two anti-angiogenic activities, one that is associated with MMP inhibition (T2N) and one that is not (T2C). These *in vitro* studies showed that the MMP-inhibitory deficient T2C is responsible for the unique ability of TIMP-2 to inhibit capillary EC proliferation. T2C inhibited angiogenesis in both the chorioallantoic membrane assay (Figure 12A) and in the mouse corneal pocket assay (Figure 13). The level of inhibition is comparable to that obtained with intact TIMP-2 suggesting that we have indeed isolated the growth-inhibitory site of TIMP-2. We further mapped the anti-proliferative activity of T2C to the 24 amino acid sequence of Loop 6 (Figure 20). Importantly, as with T2C, Loop 6 significantly inhibited angiogenesis in two different models, the CAM and the mouse corneal pocket assay (Figure 21-22). It is interesting to note that the morphology of the few remaining vessels in the CAMs treated with either T2C or Loop 6 was dramatically different than those in the CAMs treated with intact TIMP-2, in that the vessels in the

vicinity of the methylcellulose disc containing T2C or Loop 6 had a tortuous appearance and resembled vessels undergoing regression, while the CAMs treated with intact TIMP-2 were characterized by an apparent dissolution of the vessels (Figures 12, 21) (Burt et al., 1995).

The *in vivo* angiogenesis studies conducted in thesis research also reveal important differences in the types of angiogenesis inhibition effected by different domains of TIMP-2. Although the MMP-inhibitory domain, T2N, suppressed embryonic neovascularization, presumably via anti-metalloproteinase activity, this same domain did not suppress the mitogen-stimulated corneal neovascularization that most closely mimics aberrant angiogenesis in vivo. In order to determine the role of MMP inhibition alone on these two types of *in vivo* angiogenesis, we complimented our T2N studies by designing an MMP-inhibitory deficient protein in the form of EA-T2N based on recent studies demonstrating that the additional of amino acids at the N-terminus of TIMP-2 resulted in the abrogation of MMP-inhibitory activity (Wingfield et al., 1999). EA-T2N did not inhibit MMP activity and did not inhibit embryonic angiogenesis in the CAM assay (Figure 12C). In fact, although T2N resulted in, at best, modest inhibition of angiogenesis (Figure 14) in the mouse corneal pocket assay, in which neovascularization is stimulated by an angiogenic mitogen, these results are not statistically different from those observed with EA-T2N (Figure 15). In contrast, both the anti-proliferative, anti-angiogenic domain T2C and its smaller peptide, Loop 6, proved to be inhibitors of both embryonic and mitogen-stimulated angiogenesis *in vivo*. The results of this series of studies have recently been reported (Fernandez et al., 2003).

These data suggest that the inhibition of MMP activity may be sufficient to inhibit physiologic angiogenesis as represented by the embryonic vasculature of the chick chorioallantoic membrane, but alone may not inhibit the neovascularization which is characteristic of pathological conditions. This angio-inhibitory limitation may explain the less than successful results of clinical testing of synthetic MMP inhibitors (Coussens et al., 2002) whose activity is dependent solely on their enzymatic inhibition.

Section B: Loop 6 inhibits angiogenesis independent of MMP-inhibitory activity

Given that neither T2C nor Loop 6 inhibit MMP activity, our results demonstrate that TIMP-2 has an anti-angiogenic domain that is independent of direct MMP inhibition. Interestingly, a recent study has found that T2C can inhibit the activation of pro-MMP-2 presumably by sequestering pro-MMP-2 away from the activating complex (Kai et al., 2002). However, this study also demonstrates that the binding of T2C to pro-MMP-2 occurs at the carboxy-terminal tail of TIMP-2 (T2-Tail) and that these specific interactions with the PEX domain of MMP-2 are required for cell surface activation of pro-MMP-2. In fact, Kai and coworkers show that a protein comprised of Loops 4 through 6 of TIMP-2 but having the carboxy-terminal tail of TIMP-4 was unable to facilitate the activation of pro MMP-2. These findings, along with our own, demonstrate that the ability of TIMP-2 to inhibit capillary EC proliferation, which we have isolated to Loop 6 alone, is not only independent of its ability to directly inhibit MMP activity, but is also most likely to be independent of its involvement in pro-MMP activation.

In fact, our results demonstrate that the anti-proliferative effect of Loop 6 is due, at least in part, to its ability to block cell cycle progression and not to the induction of

apoptosis (Figures 24, 26). In addition, our results show that the inhibition of cell cycle progression by Loop 6 is associated with increased levels of p27 (Figure 25). These findings are not without precedent in that other inhibitors of endothelial cell proliferation have been shown to increase the level of p27 (Huang et al., 1998; Ashton et al., 1999; Goukassian et al., 2001). Our results are particularly significant, however, in that no TIMP, or fragment of any TIMP, has yet been shown to directly inhibit cell cycle progression.

The results presented here also demonstrate that T2C, and Loop 6, bind to the endothelial cell surface. It is possible that the inhibition of cell cycle progression observed is associated with a receptor-mediated event. In the last few months, Seo and coworkers have identified $\alpha 3\beta 1$ as a cell surface interacting partner with intact TIMP-2 (Seo et al., 2003). The authors suggest that the inhibition of endothelial cell proliferation may be mediated by this interaction. However, evidence for the biological consequence of the interaction between intact TIMP-2 and $\alpha 3\beta 1$ on endothelial cells was not demonstrated. Moreover, a number of recent studies have also implicated $\alpha V\beta 3$ as a putative binding protein for angiogenesis inhibitors. Given that these inhibitors differ widely in their structure and mechanism of action, it appears highly unlikely that these integrins represent cell surface receptors for these inhibitors. It remains to be determined whether Loop 6 participates in binding to this integrin, and whether $\alpha 3\beta 1$/Loop 6 is one of the complexes observed in the studies presented here. Given the number of MMP-independent activities that have been attributed to TIMPs, it is also possible that the anti-

angiogenic activity of Loop 6 is mediated by an, as of yet, unidentified cell surface receptor.

Section C: Is TIMP-4 an angiogenesis inhibitor?

One of the most challenging issues in the study of TIMP-4 presented here was related to the low yields and poor stability of TIMP-4. Although the yields are comparable to those observed by other groups when TIMP-4 was expressed in bacterial systems (Troeberg et al., 2002), it was much lower than when TIMP-4 was expressed in either mammalian or insect cell systems (Bigg et al., 1997; Liu et al., 1997). However, purification from either of these last two systems was more time consuming and involved at least 4 purification steps. Interestingly, Stratmann and coworkers have previously reported the expression of C-terminally truncated TIMP-4 (T4N) in *Pichia pastoris*. Although their yields are comparable to the ones obtained here, the cloning strategy used incorporated EA repeats at the N-terminus of the protein they expressed, similar to the MMP-inhibitory deficient EA-T2N described in Chapter 2 of this thesis. Despite the fact that the *Pichia* system can result in cleavage of these repeats, cleavage was not observed in any of the proteins produced during the course of the studies presented here, consistent with all other studies. Since incorporation of amino acid residues in at the N-terminus of TIMPs has been shown to abolish MMP-inhibitory activity, it surprising that either the EA repeats were cleaved or that TIMP-4 activity was not affected by these amino acid residues. In fact, Troeberg and coworkers found that TIMP-4 expressed in *E. coli* often contained an N-terminal methionine group which was not cleaved off after translation, and that this single

amino acid was sufficient to result in loss of MMP-inhibitory activity (Troeberg et al., 2002).

Based on the poor stability of TIMP-4, a protein purification scheme was designed to maximize the yield of pure, active protein in the least amount of time. The two-step purification scheme presented here is similar to the one used for all other proteins produced in these studies, but was empirically determined to accomplished this goal only after many other options were attempted in an effort to reduce protein degradation. In fact, other groups have remarked on the extreme difficulty of producing active TIMP-4 and of the remarkable lack of stability of this protein (Bigg et al., 2001). It is interesting to speculate that it was perhaps because of this lack of stability that the protein was never isolated. The limited expression of TIMP-4 *in vivo* coupled with the instability of the protein suggest that TIMP-4 might play a specific role in normal tissue homeostasis that may be targeted both spatially and temporally.

The results presented here demonstrate that, like other TIMPs, TIMP-4 is an inhibitor of capillary EC migration. Despite the sequence similarities to TIMP-2, however, TIMP-4 does not share anti-proliferative activity with TIMP-2 (Table V). TIMP-4 was tested for its ability to inhibit *in vivo* angiogenesis in the CAM, and was found to have no significant effect on embryonic neovascularization. It is possible that the lack of effect observed in CAMs treated with TIMP-4 could be partially due to poor protein stability, and that protein degradation in the course of the 48-hour assay resulted in loss of activity. In fact, TIMP-4 protein has never been tested in any *in vivo* assays, but has routinely been produced by either overexpression or adenoviral transformation of

	MMP Activity	Capillary EC Migration	Capillary EC Proliferation
TIMP-1	↓	↓	↑
TIMP-2	↓	↓	↓
TIMP-3	↓	↓	No Effect
TIMP-4	↓	↓	No Effect

Table V. ***In vitro*** **anti-angiogenic effects of TIMPs.** Although TIMP-4 had never been specifically tested for its ability to inhibit angiogenic processes *in vitro*, the research presented in this dissertation demonstrates that TIMP-4 is an inhibitor of capillary EC migration, but not of capillary EC proliferation, despite the sequence similarities with TIMP-2.

the cDNA in *in vivo* studies of tumor growth. It is therefore difficult to judge whether TIMP-4 would be an effective anti-angiogenic agent in a clinical setting.

Section D: Summary and future work

In the course of these studies, Loop 6 has been identified as a new inhibitor of angiogenesis found within the C-terminus of TIMP-2. Several recently identified angiogenesis inhibitors have been found to be fragments of larger parental proteins that may or may not share the activity of the inhibitor. Cryptic inhibitors of angiogenesis, including endostatin and angiostatin, are housed within proteins that do not, in their intact form, inhibit angiogenesis (O'Reilly et al., 1994; O'Reilly et al., 1997). Peptide inhibitors of angiogenesis, including the one identified here and others, such as platelet factor 4, thrombospondin and angiotensinogen, share the anti-angiogenic activity of the parent protein (REFs). It is also now widely appreciated that small molecular weight inhibitors such as Loop 6 may exhibit more desirable therapeutic potential both in terms of ease of administration and targeting as well as increased bioavailability. Loop 6 is particularly amenable to production by synthetic means as a function of its amino acid composition and small size. Moreover, since these small peptides are derived from naturally occurring proteins, they may possess the feature of reduced toxicity or other side effects and will not be rejected by the immune system (Folkman et al., 2001).

There remain a number of critical yet unsolved questions with respect to the TIMPs and their important yet diverse biological functions (Brew et al., 2000; Baker et al., 2002). Among these questions, Nagase and coworkers have highlighted the critical need for a better understanding of the structural relationship(s) between MMP inhibition,

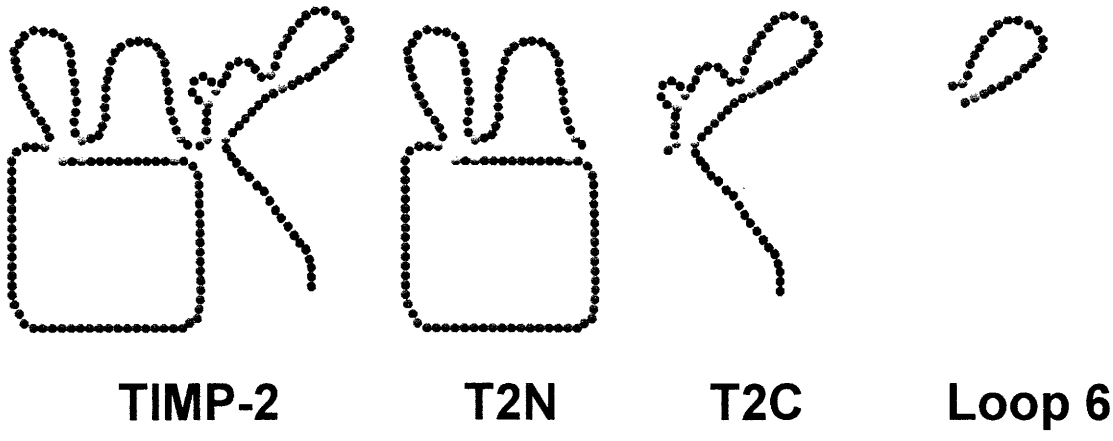


Figure 35. Uncoupling of the anti-angiogenic domains of TIMP-2. The research presented in this dissertation demonstrate that TIMP-2 possesses two structurally and functionally independent anti-angiogenic domains, T2N and T2C. T2N inhibits MMP activity and not capillary EC proliferation, while T2C inhibits EC proliferation, but not MMP activity. This research further demonstrates that the anti-proliferative activity of T2C is housed, at least in part, within Loop 6 of TIMP-2. This 24 amino acid domain of TIMP-2 is a potent inhibitor of angiogenesis *in vivo* as shown in two distinct bioassays.

cell growth-stimulating and growth-inhibiting activities, and the inhibition of angiogenesis (Brew et al., 2000). The studies conducted in this dissertation address these questions and establish the structural determinants responsible for capillary endothelial cell growth-inhibition and the inhibition of angiogenesis *in vivo* by uncoupling these activities from the inhibition of MMP activity (Figure 35). In doing so, we have discovered Loop 6, a novel, potent inhibitor of angiogenesis.

The continuing goal of this research is to understand the mechanism by which TIMPs inhibit angiogenesis. Specifically, if the inhibition of capillary EC proliferation is the result of a receptor-mediated event, the next set of experiments will address the identity of the putative receptor and the signaling cascade that might be triggered by receptor binding. Additionally, although Loop 6 is a potent inhibitor of angiogenesis, the ability of this peptide to inhibit tumor growth remains to be determined.

Chapter 8: Materials and methods

Cloning and expression of hTIMP-2 and hTIMP-2 domains

Human *TIMP-2* was cloned via PCR of a human fetal heart cDNA library (Clontech, Palo Alto, CA) using primers specific for the mature form of *TIMP-2,* the 5' primer of sequence 5'-ttc tcg aga aaa gat gca gct gct ccc cgg tgc acc cgc aac ag-3', and the 3' primer of sequence 5'-ggt cta gat caa tga tga tga tga tga tgt ggg tcc tcg atg tcg ag-3'. Two separate TIMP-2 domains were produced using PCR primers designed to yield two fragments of *TIMP-2* which encode for either the three N-terminal loops (T2N) or the three C-terminal loops (T2C). The 5' primer used to make T2N was of sequence 5'-ttc tcg aga aaa gat gca gct gct ccc cgg tgc acc cgc aac ag-3' and the 3' primer was of sequence 5'- ggt cta gat caa tga tga tga tga tga tga tgg cag ccc atc tgg tac ct-3'. The primers used to generate T2C were 5'-ttc tcg aga aaa gat gca aga tca cgc gct gcc cca tga tc-3' and 5'- ggt cta gat caa tga tga tga tga tga tgt ggg tcc tcg atg tcg ag-3'. A fourth construct, designated *EA-T2N*, was designed to produce an inactive mutant of T2N using PCR to add two amino acid residues, EA, to the N-terminus of T2N. The primers used to generate EA-T2N were 5'-ttc tcg aga aaa gag agg ctg aag ctt gca gct gct ccc cgg tgc ac-3' and 5'-ggt cta gat caa tga tga tga tga tga tga tgg cag ccc atc tgg tac ct-3'. Human TIMP-4 was also cloned from a human heart cDNA library using TIMP-4 specific primers of sequence 5'- ttc tcg aga aaa gat gca gct gct gcg ccc ctg cgc acc ctc ag-3' and 5'- ggt cta gat caa tga tga tga tga tga tga tgg ggc tga acg atg tca ac-3'. The full-length *TIMP-2* PCR product, as well as the two *TIMP-2* fragments and the mutant *EA-T2N*, were digested

with Xho I and Xba I overnight at room temperature, gel-purified using the Qiaquick Gel Purification Kit (Qiagen, Valencia, CA) and then sub-cloned into the yeast expression vector pPICZ□A (Invitrogen). Plasmids containing the genes of interest were sequenced to verify sequence fidelity and site of insertion. C-terminal His-tags were designed into each of the constructs to aid in the purification of expressed proteins. Vectors were linearized with Sac I overnight, gel purified, and then electroporated into the methylotrophic yeast *Pichia pastoris* for expression (Invitrogen). Integrants were selected by culturing on YPDS (2% peptone, 1% yeast extract, 2% glucose, 1M sorbitol, 2% agar) plates with 100□g/ml Zeocin (Invitrogen) for three days. Successful insertion of the genes of interest into the *Pichia* genome was verified by PCR using *Pichia*-specific primers, which also verified that recombination occurred at the proper site such that expression of the gene of interest is under the control of the methanol-inducible AOX1 promoter.

Four *Pichia* clones for each gene of interest were tested for expression levels, and the clone expressing the highest amount of each protein was chosen for subsequent studies. Protein identities were verified by N-terminal sequencing. Expression conditions were as follows: 25 ml overnight cultures were grown at 30° C in BMGY media (2% peptone, 1%yeast extract, 100 mM potassium phosphate pH 6.0, 1.34% yeast nitrogenous base, 1% glucose) containing 100 μg/ml Zeocin and cell pellets were collected the next day by centrifugation at 1500g. Cultures were induced by re-suspending the cell pellets in 250ml of methanol-containing media (BMMY: 2% peptone, 1%yeast extract, 100 mM potassium phosphate pH 6.0, 1.34% yeast nitrogenous base,

1% methanol), and allowed to grow for 24 hours. Media containing the secreted expressed protein was cleared of cell content by centrifugation at 3000g.

Purification of recombinant TIMP-2 and TIMP-2 domains

Expressed proteins were initially purified from the yeast media using histidine-affinity binding to a Ni-NTA Agarose resin (Qiagen) under native conditions. Briefly, expressed protein in 250 ml of cleared media was allowed to bind to 5 ml of resin by nutating for 1 hour at 4° C, and then centrifuged at low speed to collect the resin. Resin carrying the expressed protein was then loaded into a 12 ml BioRad (Hercules, CA) glass column by gravity, and the resin was washed with 15 ml of buffer containing 10 mM Imidazole (50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 10 mM Imidazole) to reduce non-specific binding. Protein was then eluted using 10 ml elution buffer containing 100 mM Imidazole (50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 100 mM Imidazole), and concentrated by centrifugation using membrane concentrators with 3 kDa molecular weight cutoff (Centriprep, Amicon). Concentrated protein was further purified to homogeneity by C4 Reverse Phase HPLC. Separation was carried out over a gradient, from 100% Buffer A (0.05% trifluoroacetic acid in water) to 60% Buffer B (0.05% trifluoroacetic acid in acetonitrile) in 60 minutes at a flow rate of 1 ml per minute. Fractions containing the peak of interest were then dialyzed versus ddH_2O to remove salts and acetonitrile. Purity was confirmed by silver staining of SDS-Page gels and/or amino acid composition. Protein was lyophilized and stored at –20° C until needed and then reconstituted in the appropriate assay buffer.

SDS-Page electrophoresis and protein sequencing

Proteins were resolved on 12% NuPage gels (Invitrogen) ran at 200V for one hour and visualized either by silver stain or coomassie. For coomassie-staining, gels were submerged in stain containing 10% isopropanol, 30% acetic acid and 0.5% Coomassie Brilliant Blue (BioRad) and allowed to nutate for 20 minutes at room temperature. Gels were then de-stained in 10% ethanol, 30% acetic acid for 1 hour. For silver-staining, gels were first fixed in buffer containing 10% methanol and 30% acetic acid for 45 minutes with shaking, then dehydrated in 50% methanol for 30 minutes. Gels were then stained with silver reagent (20 mM NaOH, 200 mM NH_4OH, 0.8% $AgNO_3$) for 20 minutes and washed with ddH_2O for 15 minutes. Proteins were visualized by developing in buffer containing 1% citric acid and 0.1% formaldehyde and the reaction stopped with 5% acetic acid.

Proteins to be sequenced were blotted onto PVDF using a BioRad Transblot Apparatus for one hour at 100V. The blots were then stained with amido black (0.1% naphtanol blue (BioRad), 10% methanol and 2% acetic acid), destained in buffer containing 45% methanol and 7% acetic acid for 15 minutes and then in buffer containing 90% methanol and 7% acetic acid for 1 minute. Protein bands were excised from the membrane with a clean scalpel blade. N-terminal sequence was determined by Edman degradation using an Applied Biosystems 477A Protein Sequencer (Dana Farber Microsequencing Facility, Boston, MA).

Peptide synthesis and purification

Peptide sequences were designed to represent various smaller structural domains of the carboxy-terminus of TIMP-2 These include: a 10 amino acid peptide corresponding to Loop 5 with sequence TRCPMIPCYI, a 24 amino acid peptide corresponding to Loop 6 with sequence ECLWMDWVTEKNINGHQAKFFACI, and a 19 amino acid peptide corresponding to the carboxy-terminal tail with sequence AWYRGAAPPKQEFLDIEDP. A fourth peptide of sequence VIRAK corresponding to a conserved sequence in the N-terminal domain of all TIMPs was also synthesized for use as a control peptide. All four peptides were synthesized via Fmoc solid phase synthesis on chlorotrityl resins using DIC/HBTU/HOBT activation on Advanced Chemtech 396-5000 multiple peptide synthesizers (ACT, Louisville, KY) to yield peptides as a trifluoroacetic acid salt. Synthesis was performed at ResGen, Invitrogen Corporation (Huntsville, AL).

Synthetic peptides were further purified by us using C18 Reverse Phase HPLC to remove any truncation products. Briefly, 1 mg of lyophilized peptide was re-suspended in 1 ml of Buffer A (0.05% trifluoroacetic acid in water), and loaded onto the column. Separation was carried out over a gradient, from 100% Buffer A to 60% Buffer B (0.05% trifluoroacetic acid in acetonitrile) in 60 minutes at a flow rate of 1 ml per minute. Fractions containing the peak of interest were collected by hand and subjected to Mass Spec analysis to confirm identity and purity. Amino acid composition analysis was used to determine yield. Purified peptides were lyophilized using a Savant speed vac and then stored at -20^{o} C.

^{14}C-labeling of type I collagen

Extracted rat tail type I collagen was allowed to dissolve in 0.01% acetic acid (2 mg/ ml) overnight at 4° C while stirring. The next morning, the pH was brought up to 8.4 with K2HPO4 and ^{14}C-acetic anhydride (10 mCi/ mmol)(1 mCi) in 1.5 ml of benzene was added dropwise over 30 minutes at 10° C while stirring. After allowing the reaction to stir for another hour, acetylated collagen was precipitated by slow addition at 4% of 30% NaCl to a final concentration of 15% NaCl. Precipitated collagen was then collected by centrifugation (27000 g for 10 minutes), resuspended in 0.5 M acetic acid, and then dialysed with 0.5 M acetic acid until no ^{14}C counts are detected in the dialysate. Labeled collagen was diluted with cold collagen dissolved in 0.5 M acetic acid to a specific activity of 10,000 CPM/15 µl and finally dialyzed in phosphate buffer (45 mM $NaPO_4$, 160 mM Na_2HPO_4, pH 7.6) and stored at 4° C.

Type I collagenase

Twenty adult bovine eyeballs were incubated in 20% betadine in PBS to reduce bacterial contamination. After 20 minutes, the eyeballs were rinsed with sterile PBS supplemented with 2% GPS and the corneas excised. Each cornea was rinsed first in 100% betadine and then twice in PBS. Once all twenty corneas were rinsed, they were minced into small pieces and incubated at 37° C for six days in 50 ml of DMEM containing 5% fetal calf serum, 1% GPS and 5 µg/ ml cytochalasin b to release type I collagenase unto the media. After six days, the media was collected, filtered and tested for collagenase activity as

described below. The collected corneas are then re-incubated with fresh media for another six days and a second collection of collagenase is prepared as described above.

MMP-inhibitory activity

MMP-inhibitory activity was assessed using a quantitative ^{14}C-Collagen Film Assay, as previously described by us (Moses et al., 1990). Briefly, 15 µl of ^{14}C-labeled collagen (10,000 CPM/ 15 µl) were added to each well of a 96 well plates and allowed to polymerize. To determine inhibitory activity, wells were treated with a known amount of activated type I collagenase plus test sample or with collagenase alone, and the plates incubated at 37°C for 2.5 hours to allow for release of ^{14}C by the enzyme. Supernantants were then analyzed in a Wallac Scintillation Counter, and percent inhibition of collagenolytic activity was calculated. An IC_{50} was defined as the amount of protein necessary to inhibit the proteolytic activity of collagenase by 50%.

Cell culture and capillary endothelial cell proliferation

Capillary endothelial cells (EC), isolated from bovine adrenal cortex, were a kind gift of Dr. Judah Folkman (Children's Hospital, Boston) and were maintained in DMEM (Gibco) supplemented with 10% calf serum (HyClone) and 3ng/ml bFGF, and grown at 37°C in 10%CO_2. Capillary EC proliferation was measured as previously reported by us (Moses et al., 1990; Moses et al., 1992; O'Reilly et al., 1994; Moses et al., 1999) using a modification of the method of Connolly and coworkers (and verified by cell counting using a Coulter Counter) (Connolly et al., 1986). Briefly, capillary EC were plated on

pre-gelatinized 96 well plates at a density of 2,000 cells per well in DMEM supplemented with 5% calf serum and allowed to attach for 24 hours. The next day, cells were treated with fresh media with or without 1ng/ml bFGF and challenged with the test proteins at various concentrations. All samples were tested in duplicate. Control wells contained cell treated with media alone or media with bFGF. After 72 hours, the media was removed and the cells were lysed in buffer containing Triton X-100 and the phosphatase substrate *p*-nitrophenyl phosphate. After a two-hour incubation at 37° C, NaOH was added to each well to terminate the reaction and cell density was determined by colorimetric analysis using a SpectraMax 190 multiwell plate reader (Molecular Devices).

Capillary endothelial cell migration

Capillary EC migration was measured using a two-chamber well system in which the upper and lower chambers are separated by a membrane with 8 μm pores through which the endothelial cells can migrate. The membranes were coated with 100 μl of 10 μg/ml fibronectin and after 1 hour 50,000 cells were plated on each of the upper chambers. The cells were allowed to attach to the membrane over 30 minutes and samples were added at various concentrations to the designated wells. Media containing serum plus bFGF was added to the lower chambers to stimulate migration. After 4 hours, the media was aspirated and the cells fixed and stained using the DiffQuik stain kit (Baxter). Briefly, cells are fixed in methanol for 5 minutes, then stained with eosin for 5 minutes to stain the nuclei, and finally stained in methylene blue for 5 minutes to stain the cytoplasm.

The wells were rinsed in water and using a cotton swab, the cells in the upper side of the membrane (non-migrating cells) were gently removed. The wells were rinsed again in water and the membranes carefully removed and mounted on glass slides using Permount mounting solution. The total number of migrated cells was determined from a digital image of each membrane. Using NIH Image, a density gradient was adjusted to mark all the dark-stained cell nuclei in the image and the total number of cells was determined electronically from the number of particles marked. Each sample was tested in duplicate.

Chick chorioallantoic membrane assay (CAM)

The chick CAM assay was conducted as previously reported by us (Moses et al., 1990; Moses et al., 1992; O'Reilly et al., 1994; Moses et al., 1999; Fang et al., 2000) Briefly, three day old chick embryos were removed from their shells and incubated in plastic Petri dishes for another three days. On embryonic day 6, samples and controls were mixed with 0.45% methylcellulose to create sample-containing discs which were then applied to the surfaces of developing CAMs, above the dense subectodermal plexus. After 48 hours of incubation, the eggs were examined for vascular reactions under a dissecting scope (60X) and photographed. All determinations were made by 3 independent members of the laboratory, in a double-blinded fashion.

Mouse corneal pocket assay

In vivo inhibition of angiogenesis was also tested using the mouse corneal pocket assay as previously described (O'Reilly et al., 1994; Moses et al., 1999). Hydron pellets containing sucrose octasulfate and, either test sample (5 □g) plus bFGF (40 ng/ml) or

bFGF (40 ng/ml) alone were implanted into corneal micropockets of C57Bl/6. Hydron pellets containing sucrose octasulfate and, either test sample (5 μg) plus bFGF (40 ng/ml) or bFGF (40 ng/ml) alone, were prepared as follows: 500 μg of lyophilized protein was resuspended in 10 μl of sodium citrate buffer (20 mM NaCi, 1mM EDTA pH 5, 9% sucrose) and mixed with 4 μg of bFGF in 10 μl of sodium citrate buffer. Alternatively, 4 μg of bFGF were diluted in 20 μl of buffer to make control pellets. Protein/ bFGF mixes or bFGF alone were then added to 10 mg of sucrose octasulfate, vortexed and allowed to dry for 4 minutes in a speed vac. The dried mix was resuspended in 10 μl of Poly(2-hydroxyethyl methacrylate) (Hydron) and spread evenly over a 10 X 10 grid on a Nytex nylon membrane with a spatula and then allowed to dry overnight. The next morning, the nylon membrane was carefully pulled apart to produce 100 hydron pellets.

Six mice per treatment group per experiment were anaesthetized and the eyeball proptosed to facilitate micropocket formation. A single surface incision was made in the cornea below the pupil and a micropocket manufactured using the end of a small spatula. Hydron pellets were then inserted into the pocket and antibiotic ointment was applied to the eye to prevent infection. Each animal carried a pellet containing the test sample plus bFGF in one eye, and a control bFGF pellet in the contralateral eye. After six days, angiogenesis was evaluated using a slit lamp microscope, and each eye was photographed. The area of neovascularization for each cornea was calculated from the length of the vessels (VL) invading the cornea as well as the clock hours (CH) covered as described by the formula VL x CH x 0.0628.

Apoptosis studies

In situ detection of cell death was determined using terminal deoxynucleotidyl transaferase dUTP nick-end labeling (TUNEL). Capillary EC were plated at a density of 10,000 cells per well on pre-gelatinized Lab-Tek Chamber Slides (Nalge Nunc, Naperville, IL) in DMEM supplemented with 5% calf serum and allowed to attach for 24 hours. The next day, cells were treated with fresh media supplemented with 1 ng/ml bFGF and challenged with an IC_{50} of Loop 6 or 100 mM wortmannin, a dose previously reported to induce apoptosis of capillary E (Flusberg et al., 2001). Cells stimulated with bFGF alone were also used as controls. All samples were tested in duplicate. After 6 hours, the cells were rinsed with PBS and then fixed in 4% paraformaldehyde for 1 hour at room temperature. Fixed cells were again rinsed with PBS and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate on ice for 2 minutes. After two more rinses in PBS, the cells were exposed to TUNEL reaction mixture (In Situ Cell Death Detection Kit, Roche Applied Science, Indianapolis, IN) and incubated at 37° C. After 1 hour, cells were then incubated with 1 μg/ml 4,6-diamidino-2-phenylindole (DAPI) at room temperature for 30 minutes, rinsed in PBS and mounted using Fluoromount G (Southern Biotechnology Associates, Birmingham, AL). Apoptotic nuclei were detected using a Nikon Eclipse TE300 microscope (Nikon Instruments, Melville, NY).

Cell cycle analysis

Capillary EC were plated at a density of 100,000 cells per plate unto pre-gelatinized 60 mm dishes in DMEM supplemented with 5% calf serum and allowed to attach overnight.

The next day, the media was replaced with DMEM supplemented with 0.4% calf serum in order to synchronize the cells by serum starvation. After 36 hours, synchronized cells were stimulated with DMEM containing 5% calf serum plus 1 ng/ml bFGF, and then treated with an IC_{50} of Loop 6 or an equivalent dose of the T2-Tail peptide. After 16 hours, the cells were rinsed with PBS, trypsinized and collected by centrifugation. Pelleted cells were rinsed again with PBS and resuspended in 1 ml of 80% ethanol in PBS, and stored at –20° C overnight. The next morning, cells were collected by centrifugation and resuspended in 500 µl of staining solution (0.1% sodium citrate, 100 µg/ml RNAse A, 0.1% Nonidet-P40, 50 µg/ml propidium iodide). After 30 minutes, DNA content and the percentage of cells in the various stages of the cell cycle were determined using a FACSVantage SE (Becton Dickinson, Franklin Lanes, NJ). All samples were tested in duplicate. Histograms of representative experiments for each test condition were created using WinMDI 2.8 (The Scripps Research Institute, La Jolla, CA).

Western analysis

Capillary EC were plated, synchronized and treated with an IC_{50} of Loop 6 as described above for cell cycle analysis. After 16 hours, cells exposed to bFGF alone or bFGF and Loop 6 were rinsed in PBS and lysed directly on the culture dish by addition of 500 µl of lysis buffer (1% SDS in 10 mM Tris-HCl, pH 7.4). Collected lysates were boiled for 5 minutes, mixed by vortexing and then placed on ice. Samples were repeatedly passed through a 22-gauge syringe needle in order to shear the DNA in the samples. The samples

were then centrifuged, and the supernatant collected. Protein concentration of the lysates was determined using the MicroBCA method (Pierce, Rockford, IL).

Equal amounts of protein were loaded unto 12% SDS-PAGE gels under reducing conditions, resolved by electrophoresis and subsequently transferred to nitrocellulose using a TransBlot apparatus (BioRad). Membranes were blocked with 5% low fat dry milk in TBST (10 mM Tris, pH 7.2, 50 mM NaCl, 0.5% Tween 20) overnight at 4° C and then probed for 1 hour at room temperature with antibodies to various cell cycle associated proteins, including: Cyclin D1 (Pharmingen, BD Biosciences, Palo Alto, CA), Cyclin E (Pharmingen), Cyclin A (Santa Cruz Biotechnology, Santa Cruz, CA) and p27 (Santa Cruz). Blots were washed three times with TBST and then incubated with a 1:5000 dilution of either mouse or rabbit horseradish peroxidase conjugated secondary antibodies (Sigma, St. Louis, MO) for 30 minutes at room temperature. Labeled proteins were detected using Supersignal West Pico Chemiluminescence Substrate (Pierce).

Iodination and protein crosslinking

Protein labeling with ^{125}I was performed using IODO Beads Reagent (Pierce) following manufacturer's instructions. Briefly, one IODO bead was mixed with 5 μl $Na^{125}I$ (100 mCi/ ml) and 95 μl of 100 mM $NaPO_4$, and allowed to react for 5 minutes. 5 μg of test protein in 40 μl of 100 mM $NaPO_4$ was then added and allowed to react with the charged bead for an additional 5 minutes. The labeled protein was then separated from free label using a G-25 Sepharose size exclusion column and collecting 250 μl fractions. Fractions were then counted on a gamma scintillation counter to identify the fractions containing

labeled protein. Relative yield and specific activity was then calculated for the polled fractions containing labeled protein.

For crosslinking studies, capillary EC were plated on 10 cm dishes and allowed to reach 60% confluency. Cells were then placed on ice, washed with cold PBS, and then incubated in 2 ml binding buffer (DMEM supplemented with 0.1% gelatin and 20 mM HEPES) plus test ^{125}I–labeled protein for 1.5 hours on ice. Interacting proteins were crosslinked using 0.128 mM Bis(Sulfosuccinimidyl) suberate (BS^3) (Pierce) for 15 minutes on ice. The cells were rinsed with PBS three times to remove any unbound protein. Cells were scraped off the culture dishes using 500 µl of 2.5mM EDTA, pH 8.0 in PBS, collected and pelleted at 14,000 g for 1 minute. The collected cell pellets were then resuspended in 25 µl of lysis buffer (10 mM EDTA, pH 8.0, 1% nonidet, and 1X protease inhibitor cocktail (Roche)) and incubated on ice for 15 minutes. After 15 minutes, the lysates were again spun at 14,000g for 1 minute and the supernatant collected in a clean tube. The cellular membrane fraction was boiled for 5 minutes and then resolved by SDS-PAGE. Autoradiography of the gel was used to determine the presence and molecular weight of putative receptor/TIMP-2 complexes.

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